Brian K. Nunnally · Vincent E. Turula
Robert D. Sitrin *Editors*

Vaccine Analysis: Strategies, Principles, and Control

Springer

Vaccine Analysis: Strategies, Principles, and Control

Brian K. Nunnally · Vincent E. Turula
Robert D. Sitrin
Editors

Vaccine Analysis: Strategies, Principles, and Control

Springer

Editors
Brian K. Nunnally
Apex, NC
USA

Vincent E. Turula
Pfizer BioTherapeutics Pharmaceutical Sciences
Pearl River, NY
USA

Robert D. Sitrin
Sitrin Solutions, LLC
Lafayette Hill, PA
USA

ISBN 978-3-662-45023-9 ISBN 978-3-662-45024-6 (eBook)
DOI 10.1007/978-3-662-45024-6

Library of Congress Control Number: 2014956187

Springer Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer-Verlag GmbH Berlin Heidelberg is part of Springer Science+Business Media (www.springer.com)

Foreword

Infections and their associated diseases are arguably the most significant environmental factors impacting individual health, as well as the overall health and well-being of populations. The successful development and implementation of interventions to prevent and treat infections over the past two centuries have had a profound positive effect on human and animal health. Among these interventions, the use of vaccines in healthy people for prevention of infection has proven to be one of the most effective, from both public health and economic perspectives. Vaccination has resulted in the eradication of smallpox and, in many parts of the world, substantial control of deadly diseases such as measles, polio, pneumococcal pneumonia, tetanus, diphtheria, rotaviral diarrhea, and hepatitis B. There are numerous other infectious diseases to add to this list, but the control and elimination of these alone have yielded substantial improvements in the quality and quantity of life.

Given this, understanding the technical challenges in vaccine development, manufacture, and supply is critical to assure the continuous availability of existing vaccines and to facilitate the introduction of new vaccines. Vaccines are complex biological products that encompass a full spectrum of entities from whole infectious attenuated organisms to chemically and genetically modified proteins and polysaccharides. The structural and formulation complexities of vaccines are extraordinary, and are further amplified by the unique fact that vaccines have no inherent biological activity other than the ability to elicit an effective immune response in the recipient. Accordingly, a thorough analytical understanding is essential to assure a vaccine's biological consistency as it undergoes its developmental transition from the research laboratory to manufacturing scale. Detailed analytical understanding is also essential to assure a vaccine's consistency of production and effectiveness over years of use. In the end, analytical understanding is fundamental to the maintenance of an effective vaccine supply.

The central role of analytical methods and strategies in vaccine development and production is reflected in the chapters of this volume. The first section focuses on a comprehensive description of analytical approaches and methods associated with

existing vaccine modalities, including analytical considerations during late stage development, manufacturing and commercial control. The second section provides guidance on the strategic use of analytical vaccine assessment, detailing experiences regarding quality control, regulatory requirements, production control, and expanded distribution requirements. It is the hope of the editors and contributing authors of *Vaccine Analysis* that this volume will provide a unique and comprehensive single point of reference for the analytical control strategies essential to the continued successful development and use of vaccines.

As a research scientist who has spent over 30 years involved with the study of infectious diseases and with the development of successful vaccines, I have deep respect and appreciation of the role that analytical scientists play in successful vaccine development and manufacture. The experiences detailed herein will serve as a foundation of knowledge for the vaccine developers of the future.

Emilio A. Emini Ph.D.
Senior Vice President and Head
Vaccine Research and Development
Pfizer Inc.

Preface

For those of us in vaccine analytical and process development, it was apparent that a central resource on analytics would be helpful, especially since many new organizations both in the developed and developing markets are working on new vaccine products. We thus solicited input from a variety of analytical leaders to put together this volume on analytics of vaccines. Historically, vaccines were relatively crude preparations and quality control testing was primarily focused on safety issues such as sterility and absence of adventitious agents as well as a potency testing as a surrogate for clinical performance. With the advent of more modern technology applied to biotech products, testing and release for many modern vaccines are carried out by sophisticated techniques such as Mass Spectrometry, PCR, NMR, HPLC, and Flow cytometry.

Vaccines have had a profound impact on preventing or eliminating many major diseases worldwide. Since vaccines are administered to healthy individuals, it is critical that their safety and efficacy be under strict control by carefully constructing the manufacturing process and the analytical Quality Control packages. Unlike low molecular weight drugs or modern biologics, vaccines have always been considered to be complex products manufactured by complex processes. This has manifested itself in strict control of the manufacturing process often expressed as "the process defines the product." Corresponding quality control packages are an important part of the release paradigm based on classical safety technology and a potency assay developed at the time of licensure and correlated with clinical efficacy. Although this paradigm still exists today, the application of modern biotechnology analytical methods have opened up vaccine products for further characterization. Nevertheless, the diversity of vaccine products requires the application of a wide array of analytical and QC technologies.

The claim that a vaccine is *Safe* and *Efficacious* can only be asserted through proper control with suitable and capable analytical methods. This necessity was realized over 100 years ago when Milton Joseph Rosenau was the first to test mass produced Small Pox vaccine for potency and purity. (Willrich 2011) During the

twentieth century, as regulation, production fostered a shift from inactivated whole cell vaccines to purified subunit based vaccines because of enhanced safety profiles (Plotkin and Plotkin 2011; Zhao et al. 2013). Combination vaccines that contain multiple antigens required rigorous demonstration of maintenance of the individual components to confer lasting immunity (Galambos and Sewell 1995; Galambos and Sturchio 1996). The testing paradigm has not changed. However, the field is experiencing a shift in needs—rapid response for rapidly emerging Influenza epidemics, flexibility in location of manufacture, horizontal, and philanthropic vaccine production where individual components are furnished by unique entities either academic, government, or industry. Regardless of the evolved development of the field, the need for safety and efficacy remains paramount but their demonstration will require sound analytical control. Sound analytical control fulfills both regulatory oversight, e.g., ICH Q2 guidances and business needs by providing accurate and precise monitoring of critical structural elements from cell bank to drug substance and final drug product dosage form.

This book can serve as a reference for vaccine control for current and future vaccine platforms. The chapters in the book describe release testing and characterization technology applied to commercial vaccines as well as vaccines in late stage clinical development. The editors in conjunction with the contributors structured the book to detail analytical control for all current vaccine modalities. In addition, current topics that are supplemental to the actual implementation of an analytical control and that are confronting vaccine quality control and regulatory organizations are developed; and thus provide a vantage point for those in such endeavors. For these sections, authors offer a trajectory of guidance from experiences as vaccine production; distribution and market expand globally beyond established markets in the United States and Europe in the twenty-first century. Adjuvants, a critical component of most vaccine formulations (and delivery) were not included given their broad scope and mechanisms of immune stimulation and that many other excellent reviews exist at this time. Despite increasing technological improvements the need to have sound analytical control in clinical stages development for the maturation of process and formulation into reliable commercial control to assess safety and efficacy remain. The comprehensive descriptions of each chapter describe how analytical methodologies are employed and structured to support process control, release, and stability. As the intentions of the editors, *Vaccine Analysis*, offers a unique, single point of reference to capture analytical control strategies for all primary vaccine modalities.

Brian K. Nunnally
Vincent E. Turula
Robert D. Sitrin

References

Galambos L, Sewell JE (1995) Networks of innovation: Vaccine development at Merck, Sharp & Dohme, and Mulford. Cambridge University Press, New York

Galambos L, Sturchio JL (1996) The pharmaceutical industry in the twentieth century: A reappraisal of the sources of innovation. Hist Technol 13(2):83–100

Plotkin S, Susan Plotkin S (2011) The development of vaccines: How the past led to the future. Nat Rev Microbiol 9:889–893

Willrich M (2011) Pox: An American history, 1 edn. Penguin Press HC, New York

Zhao Q, Li S, Yu H et al. (2013) Virus-like particle-based human vaccines: Quality assessment based on structural and functional properties. Trends Biotechnol 31(11):654–663

Contents

Chapter 1
Live Attenuated Viral Vaccines

Mark S. Galinski, Kuldip Sra, John I. Haynes II and Jennifer Naspinski

1.1 Introduction

1.1.1 Live Viral Vaccine History

The history of vaccination using live viruses extends through several centuries and is directly intertwined with the elimination of smallpox (variola major, Plotkin and Plotkin 2004). The word vaccine comes from the Latin "vaccinus" meaning "of cows"—an adjective joining variola (smallpox) and vaccinae to form cowpox (variola vaccinae, Jenner 1798; Riedel 2005) as distinct from the human disease (variola). Variola was easily transmitted, had high mortality, and for those who recovered, resulted in significant disfigurement due to scarring.

Inoculation of variola (pustule fluids or dried scabs) had been used in Eastern countries, including China, since the late seventeenth century (Henderson and Fenner 1994; Hong and Yun 2001). This practice, termed variolation, had significant uncertainty in outcome in that the inoculated recipient could have a mild or fulminate disease leading to death. Despite the uncertainty, variolation was attributed with reducing smallpox deaths, which might run as high as 30 %, to a more "acceptable" level of 2–3 % (Henderson and Fenner 1994).

Although the importance of variolation to prevent smallpox was subsequently transmitted to Western practitioners, it was the work of Edward Jenner in the late

M.S. Galinski (✉)
Integrative BioConsulting, LLC, 2485 Richland Ave,
San Jose, CA 95125, USA
e-mail: mark.galinski@integrative-bc.com

M.S. Galinski · K. Sra
MedImmune, Gaithersburg, USA

J.I. Haynes II · J. Naspinski
Merck & Co., Inc., West Point, PA 19486, USA

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6_1

eighteenth century that demonstrated the effectiveness of variolation with cowpox (subsequently termed "vaccination" by Louis Pasteur (Pasteur 1881a)) to prevent smallpox (Jenner 1798). Vaccination with cowpox to prevent smallpox continued well into the late twentieth century with no significant technological improvement in the preparation of the vaccine inoculum, which was still generated in a whole animal (indeed Dryvax® was still used by the military until 2008 (Nalca and Zumbrun 2010)). Nonetheless, with vigilant vaccination programs being adopted worldwide, smallpox was eradicated, and the world was declared smallpox free by the World Health Organization (WHO) in 1979 (World Health Organization 1979, 1980).

Vaccination for the prevention of smallpox relied upon the chance observation that a less virulent (attenuated) form of a disease could provide protection against the virulent form (Jenner 1798). While smallpox was amenable to the development of an effective vaccine (cow pox), further development of antiviral vaccines stalled until the late nineteenth century. At this time, Robert Koch (Carter 1985) formulated the germ theory concept of disease, and Louis Pasteur (Pasteur 1881a) observed that attenuation of virulent microorganisms was possible.

Vaccine development subsequently progressed primarily in the realm of antimicrobial vaccines. In the late nineteenth century, Louis Pasteur demonstrated that exposure to an attenuated culture of the bacteria responsible for chicken cholera (*Pasteurella multocida*) protected chickens from the fulminate disease when they were later inoculated with the virulent bacteria (Pasteur 1881a, b). This landmark discovery demonstrated attenuation could occur in culture, albeit following unknown biological changes. Together with Koch's germ theory (that a disease was casually associated with a specific microorganism), this discovery opened the path to further development of vaccines against specific microorganisms.

In the 1880s, Pasteur and Emile Roux developed the first rabies vaccine (Pearce 2002; Théodoridès 1989). The vaccine was derived from rabies virus that had been passaged in rabbit brains and the harvested material was dried as a means of "attenuation." This vaccine was first used in 1885 on a young boy who had been bitten by a rabid dog, and found to be effective.

While the fundamental principles for vaccines and passive immunity (antitoxins) were well established by the beginning of the twentieth century, limitations in technology prevented significant improvements in vaccine development until the mid-twentieth century when viruses could be isolated and propagated in eggs and subsequently in cell culture.

The third human vaccine developed after smallpox and rabies was to yellow fever. In 1932, a vaccine prepared in mouse brain was developed; however, this vaccine required simultaneous injection of human immune serum to prevent adverse events (Norrby 2007; World Health Organization 1956). A modified strain was developed subsequently that did not require administration of immune serum; however, it continued to be cultured in mouse brain and had neurotropic properties that resulted in a high incidence of encephalitis reactions.

In 1937, Max Theiler showed continuous passage of yellow fever virus in vitro resulted in changes to its pathogenicity that were dependent upon the tissue used (Theiler and Smith 1937). When cultured in chick embryos, the virus lost its

virulence and induced a long-lasting protective immunity in humans. The work on the yellow fever vaccine was pioneering in that it established a model for the scientific, technical, and regulatory approaches for continued vaccine development for the remainder of the twentieth century (Frierson 2010; Monath 1996; Norrby 2007), including:

- Replacement of an animal (mouse) with primary tissue (chick) culture as a manufacturing substrate for vaccine production,
- Sequential passage of virus in tissue culture to select naturally (spontaneously) occurring attenuated vaccine stocks,
- Importance of seed lot system (stock seeds) for vaccine production that
  - Ensures vaccine lots of consistent potency, safety, and efficacy,
  - Prevents production of under-attenuated or over-attenuated lots,
  - Reduces adverse events.
- Acceptance of significant medical risk (adverse reactions) to protect from disease (mouse cultured vaccine discontinued in 1980 (Frierson 2010)).

1.1.2 Live Vaccine Categories

A live vaccine is a microorganism (microbial or viral) that can replicate in the host or can infect cells and function as an immunogen without causing natural disease. While there are live vaccines that use attenuated bacterial organisms including *Mycobacterium bovis* strain Bacillus Calmette–Guérin (BCG) to protect against tuberculosis, *Salmonella typhi* strainTy21a to protect against typhoid, and *Orientia tsutsugamushi* strain E to prevent scrub typhus (Killeen and DiRita 2001; Roland et al. 2005; Valbuena and Walker 2013), the majority of live vaccines are live attenuated viral vaccines, and it is these vaccines that will be focus of the rest of this chapter.

1.1.3 Live Attenuated Virus Vaccines

Live attenuated virus vaccines are used to protect against the diseases listed in Table 1.1. This table indicates the year of introduction and virus component valency (number of serotypes or subtypes used).

The following steps outline how a live attenuated virus vaccine protects an individual from infection or disease (Greenberg and Arvin 2011; Plotkin 2003, 2008; Siegrist 2008):

- The vaccine introduces a nonharmful (attenuated) form of the pathogen into the body by injection, oral administration, or scarification,

Table 1.1 Live attenuated virus vaccines and year of introduction

Year	Disease	Valency
1798[a]	Smallpox (cowpox)	Monovalent
1885[a]	Rabies	Monovalent
1935	Yellow fever	Monovalent
1957	Adenovirus (respiratory)	(2) Serotypes
1962	Polio vaccine (oral vaccine)	(3) Serotypes
1963	Measles	Monovalent
1967	Mumps	Monovalent
1969	Rubella	Monovalent
1971	Combined MMR (measles, mumps, and rubella)	Trivalent
1974	Varicella (chickenpox)[b]	Monovalent
1998[c]	Rotavirus	(1) Rhesus serotype (3) human/rhesus reassortants
2003	Influenza (trivalent)	(3) Serotypes/subtypes
2005	Combined MMRV (measles, mumps, rubella, and varicella)	Quadrivalent
2006	Rotavirus	(5) Serotypes (human/bovine reassortants)
2006	Zoster (shingles)[b]	Monovalent
2008	Rotavirus	(1) Serotype (human)
2010	Japanese encephalitis	Monovalent (Japanese encephalitis virus/yellow fever virus chimera)
2012	Influenza (quadrivalent)	(4) Serotypes/subtypes

[a] Year of assessment in a human subject

[b] Varicella and zoster are caused by the same virus, Herpes varicella-zoster. The varicella vaccine is dosed to prevent chickenpox in children, whereas the zoster vaccine is dosed in adults to prevent shingles, a sequelae following previous chickenpox infection. Both vaccines utilize the same virus

[c] A rotavirus vaccine (RotaShield, attenuated rhesus and human/rhesus reassortants) was first introduced in 1998 by Wyeth, but the product was voluntarily withdrawn from the market in 1999 due to an association with intussusception (Centers for Disease Control and Prevention (CDC) 1999; Murphy et al. 2001))

- Replication of the attenuated virus occurs, although with insufficient robustness to result in clinical disease. This replication results in amplification and subsequent presentation to the immune system, which is required for the vaccine to be effective,
- The immune system in the body recognizes specific vaccine antigen(s) and produces an immune response to the vaccine by making antibodies, killer cells or both,
- When the pathogen is naturally encountered, the immune system remembers the vaccine-presented antigen due to the presence of memory B cells (producing antibodies) and memory T cells (helping the production of antibodies or killer T cells) which mount a much larger and quicker response than would have happened if the person had never received the vaccine. This is called "immune memory," and

- Vaccination can induce immunity in several ways to fight infection and/or disease:
  - By immediately neutralizing the pathogen due to preexisting vaccine-induced antibodies,
  - By impacting replication of the pathogen, reducing its replication efficiency significantly, thereby allowing the immune system to eliminate the pathogen,
  - By inducing the production of more antibodies that further protect against disease from the pathogen (antibody response), and
  - By inducing immune cells to recognize and eliminate infected cells and/or to release cytokines that further enhance immune clearance of the pathogen.

Many live attenuated viral vaccines were initially developed as monovalent vaccines (e.g., smallpox, yellow fever, measles, mumps, rubella, and varicella) or as mixtures of multiple serotypes (e.g., adenovirus, influenza virus, poliovirus, and rotavirus). Combination vaccines consisting of different virus types were introduced to reduce the number of vaccinations. Thus, measles, mumps, and rubella initially were introduced as monovalent vaccines and eventually were combined to make a trivalent vaccine M-M-R® in 1971. The trivalent vaccine was further expanded into a quadrivalent vaccine containing a varicella component (ProQuad®) in 2006.

Table 1.2 lists live viral vaccine product names, trade names, and sponsors (license holders). This list is not inclusive of all licensed vaccines worldwide, but does illustrate the breadth of products available.

1.2 Common Issues for Live Attenuated Viral Vaccines

Live attenuated viral vaccines are intended for people who have not yet been naturally infected, with the exception of ZOSTAVAX® (for the prevention of shingles in adults previously infected with chickenpox). These vaccines prepare the immune system to respond in case of future exposure to the pathogen, and are routinely administered to healthy babies, children, adults, and seniors with the goal of preventing disease. As such, these vaccines differ from all other medicines in that they do not treat a preexisting disease and are administered to healthy recipients. In addition, they are generally administered to large numbers of the population worldwide. Requirements for vaccines include:

1. *Safety*—does not cause adverse side effects (clinical disease, encephalitis, inflammation, high fever, nontransmissible, long-term sequelae, etc.),
2. *Efficacy*—is able to reduce disease in the field,
3. *Stability*—can be stored at acceptable temperatures (2–8 °C or room temperature) for a defined period of time (6 months to 2 years) and maintain acceptable potency to effect a response, and
4. *Reasonable Cost*—able to be produced in sufficient quantities and affordable to meet the needs of the intended population.

Table 1.2 Live attenuated virus vaccines

Product name	Trade name	Sponsor
Adenovirus type 4 and type 7 vaccine, live, oral	No trade name	Barr Labs, Inc.
Measles, mumps, and rubella virus vaccine, live	Priorix®	GlaxoSmithKline Biologicals
Measles virus; rubella virus; mumps virus; varicella vaccine—live	Priorix-Tetra®	GlaxoSmithKline Biologicals
OPV (oral polio vaccine) bivalent types 1 and 3	Polio Sabin One and Three®	GlaxoSmithKline Biologicals
OPV (oral polio vaccine) monovalent type 1	Polio Sabin Mono T1®	GlaxoSmithKline Biologicals
OPV (oral polio vaccine) monovalent type 2	Polio Sabin Mono Two (oral)®	GlaxoSmithKline Biologicals
OPV (oral polio vaccine) monovalent type 3	Polio Sabin Mono Three (oral)®	GlaxoSmithKline Biologicals
OPV (oral polio vaccine) trivalent	Polio Sabin®	GlaxoSmithKline Biologicals
Rotavirus vaccine, live, oral	ROTARIX®	GlaxoSmithKline Biologicals
Varicella virus vaccine, live, attenuated (oka-strain)	VARILRIX®	GlaxoSmithKline Biologicals
Influenza A (H1N1) 2009 monovalent vaccine	No trade name	MedImmune, LLC
Influenza vaccine, live, intranasal (trivalent, types A and B)	FluMist®	MedImmune, LLC
Influenza vaccine, live, intranasal (quadrivalent, types A and types B)	FluMist® Quadrivalent	MedImmune, LLC
Measles, mumps, and rubella virus vaccine, live	M-M-R®II	Merck & Co, Inc.
Measles, mumps, rubella, and varicella virus vaccine live	ProQuad®	Merck & Co, Inc.
Varicella virus vaccine live	VARIVAX®	Merck & Co, Inc.
Rotavirus vaccine, live, oral, pentavalent	RotaTeq®	Merck & Co., Inc.
Zoster vaccine, live (Oka/Merck)	ZOSTAVAX®	Merck & Co., Inc.
Attenuated, monovalent oral poliomyelitis vaccine type 3 (sabin)	Mono OPV3®	Novartis Vaccines & Diagnostics
Live, attenuated, bivalent oral poliomyelitis vaccine type 1 and type 3	Bivalent OPV (type 1 & 3)®	Novartis Vaccines & Diagnostics
Live, attenuated, monovalent oral poliomyelitis vaccine type 1 (sabin)	Mono OPV1®	Novartis Vaccines & Diagnostics
OPV (oral polio vaccine) trivalent	Polioral®	Novartis Vaccines & Diagnostics
Japanese encephalitis vaccine (live, attenuated)	IMOJEV®	Sanofi Pasteur Biologics Co.

(continued)

Table 1.2 (continued)

Product name	Trade name	Sponsor
Measles virus, live	ROUVAX®	Sanofi Pasteur Biologics Co.
Measles, mumps, and rubella	TRIMOVAX MÉRIEUX®	Sanofi Pasteur Biologics Co.
OPV (oral polio vaccine) bivalent type 1 and 3	Oral bivalent types 1 and 3 poliomyelitis vaccine	Sanofi Pasteur Biologics Co.
OPV (oral polio vaccine) monovalent type 1	Oral monovalent type 1 poliomyelitis vaccine	Sanofi Pasteur Biologics Co.
OPV (oral polio vaccine) trivalent	OPVERO®	Sanofi Pasteur Biologics Co.
Smallpox (vaccinia) vaccine, live	ACAM2000®	Sanofi Pasteur Biologics Co.
Yellow fever	STAMARIL®	Sanofi Pasteur Biologics Co.
Yellow fever vaccine	YF-Vax®	Sanofi Pasteur Biologics Co.

Excerpted from the US Food and Drug Administration, complete list of vaccines licensed for immunization and distribution in the US. http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM093833 and the World Health Organization, WHO prequalified vaccines. http://www.who.int/immunization_standards/vaccine_quality/PQ_vaccine_list_en/en/

Generally, live attenuated viral vaccines are not rigorously purified, and terminal sterile filtration is not performed during drug product manufacture. Accordingly, aseptic manufacture using well-characterized substrates (cells, embryonated eggs), raw materials, starting materials, and intermediates of known origin and quality form the foundation for assuring the purity and safety of the vaccine product.

Analytical testing, as described further below, focuses on safety testing and sterility testing to assure the absence of extraneous agents (microbial and viral), and includes specific tests which monitor for the removal of process residuals (e.g., bovine serum, egg ovalbumin, Benzonase®, EDTA, etc.) to levels that are accepted by regulatory authorities.

1.3 Biologics Chemistry, Manufacturing and Control (CMC): General Considerations

Live attenuated viral vaccines consist of live viruses that are developed from wild type viruses by eliminating their virulent properties. Attenuating mutations can be introduced using a variety of different approaches. Traditionally, most live attenuated viral vaccines (e.g., smallpox, yellow fever, poliomyelitis, measles, mumps, rubella, varicella, and influenza) were based on selection of naturally occurring

variants (mutants) that occurred during passage of the virus in semi-permissive or atypical animal cells (or animals) and/or under altered growth conditions. The naturally occurring variants were attenuated and found to have reduced capabilities to cause disease in their original host.

As an example, the measles vaccine virus (Moraten) was attenuated by extensive passage in human kidney cells, human amnion cells, chicken embryo (embryonated eggs), and chicken embryo fibroblasts. Further, passaging under reduced temperatures (32 °C for 40 passages) in chick embryo fibroblasts was performed to eventually select a more attenuated vaccine strain called the Moraten strain (Parks et al. 2001).

Another means for introduction of attenuation (specific to segmented RNA viruses) is to make reassortant chimeras consisting of the immunologically relevant virus segments (i.e., human rotavirus capsid genes) combined with attenuating virus segments (i.e., bovine rotavirus segments). Indeed, the influenza vaccine virus is generated each year using recombinant reverse genetics to construct the vaccine virus containing the immunologically relevant virus segments (hemagglutinin and neuraminidase glycoprotein genes) with the attenuating virus segments.

Characteristics of the strains suitable as vaccine viruses should meet the following criteria:

- Ability to replicate and induce protective immunity,
- Inability to cause disease, and
- Genetic stability such that the strains do not revert to pathogenic forms after multiplication in the human host.

Regardless of the methods used for derivation of the attenuated virus, the resulting vaccine viruses are able to replicate and are fully immunogenic. Live attenuated viruses induce more durable and prolonged immunological responses and thus are preferred for vaccinating healthy individuals.

Figure 1.1 shows electron micrographs (EM) of adenovirus, influenza, mumps, rotavirus, and varicella-zoster. It should be noted that the LVV active components are complex entities (organisms) and not simple biochemical entities. Although the drug product is comprised of a complex mixture of excipients and drug substance (viable vaccine virus, inactivated virus antigen, process residuals), it is the viable vaccine virus that replicates in the recipient that induces an appropriate immunity.

Since live attenuated viral vaccines are grown in animal or human cells, contamination of the drug substance with the cell substrate (intact cells), extraneous virus (es), or other microbial agents already present in these substrates is a potential risk that requires significant manufacturing controls and testing to assure vaccine safety.

Because LVV products require virus replication to induce protective immunity, the infectivity of the vaccine virus (as measured by potency) must be maintained. However, most vaccine viruses are prone to losing infectivity during the manufacturing of the drug substance and drug product and following distribution to the market. For viruses with membranes (e.g., measles, mumps, rubella, varicella, influenza), the virus particles degrade and viral membranes get disturbed. Figure 1.2 shows an electron micrographic image of influenza virus particles after processing and purification steps.

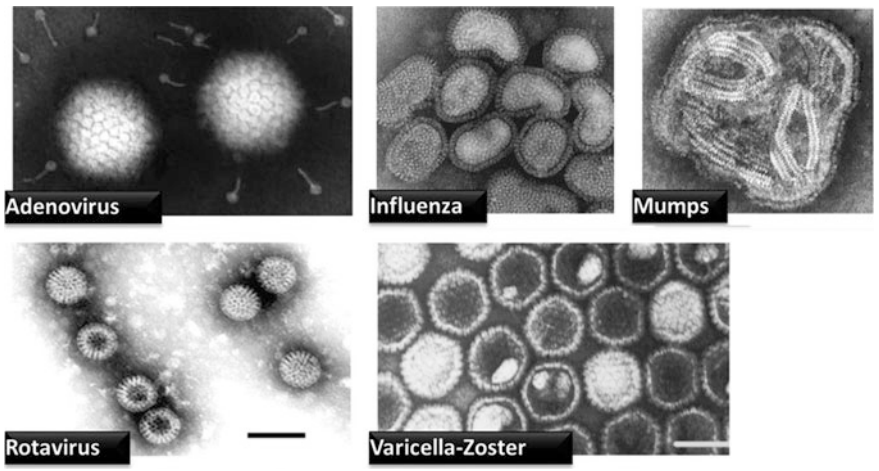


Fig. 1.1 EM images of representative viruses for which live attenuated viral vaccines have been developed for prevention of disease [Sander, D.M. http://virology.net/Big_Virology/BVHomePage.html (accessed April 21, 2014). The big picture book of viruses: virus families by genome type]

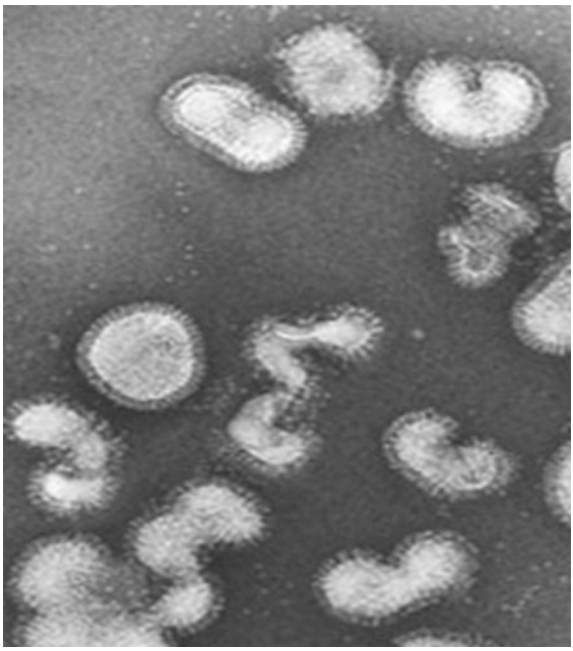


Fig. 1.2 Negative stain electron microscopy image of H1N1 (influenza) vaccine at 52,000 × magnification after harvest, processing and purification [www.NanoImagingServices.com (accessed April 21, 2014). Characterization of viruses and vaccines: Adeno5 and H1N1; image courtesy MedImmune]

The picture shows intact and spindle shaped forms of virus particles. The virus membrane is deteriorated and is broken in many particles. In addition, some particles have fused virus membranes. Because of the inherent fragility of many viruses, processing and purification of the LVV products are very gentle in nature.

1.4 Biologics CMC: Raw Materials and Starting Materials

Many raw materials and starting materials are used in the synthesis of an LVV product intermediate, drug substance, or drug product.

Raw materials used in vaccine manufacturing generally consist of the culture media, buffers, and bioreactor components used for the growth and propagation of the cell substrates. Culture media and buffers are assumed to contain defined biochemicals, inorganic salts, and/or growth factors. However, more complex biological materials such as trypsin, bovine serum, human serum albumin, and gelatin (or other hydrolysates) may also be used. They may be incorporated into the drug substance, they may impart a stabilizing effect, or they may be added as excipients.

Starting materials consist of the cell substrates and virus seeds (master virus seed and working virus seeds). The cell substrate material may be as complex as an embryonated egg or primary cells derived from a tissue (e.g., chick embryo fibroblasts, monkey kidney cells), or as controlled as a cell line (e.g., MRC-5, WI-38, Vero) cultured in a chemically defined cell culture medium. The starting material may be used for the cultivation of the cell substrate or used for manufacture of the drug substance.

All raw materials and starting materials have stringent quality requirements for use in vaccine manufacturing; however, it should be noted that because of the nature of the manufacturing process (absence of any inactivation process, and no terminal sterile filtration), safety testing at critical manufacturing steps forms the basis for most of the analytical testing. Manufacturer changes to key raw materials must also be monitored to ensure there is no impact to the product, which may require demonstration and qualification of the material in the process.

1.5 Biologics CMC: Cell Substrate and Analytical Methods

The cell substrate used for vaccine manufacturing is considered a starting material. In general, most live virus vaccines are manufactured with cultures of cells that have been derived from primary tissues (e.g., chicken sourced embryonated eggs, primary chick embryo fibroblasts) or from manufacturing cell banks (e.g., MRC-5, WI-38, FRhL-2, Vero). It is critical that these materials be fully assessed to demonstrate their safety and freedom from extraneous agents (microbial, viral, prions) (European Pharmacopoeia (Ph. Eur.), EP 5.2.3; European Pharmacopoeia (Ph. Eur.), EP 5.2.8; FDA Guidance for Industry 2010; United States Pharmacopeia and The National Formulary, USP <1235>; WHO 2007).

Table 1.3 Pathogens tested for in SPF flocks

Specific pathogen-free agent (European Pharmacopoeia (Ph. Eur.), EP 5.2.2)	
Avian adenoviruses group 1	Infectious bursal disease virus
Avian encephalomyelitis virus	Influenza A virus
Avian infectious bronchitis virus	Marek's disease virus
Avian infectious laryngotracheitis virus	Newcastle disease virus
Avian leucosis viruses	Turkey rhinotracheitis virus
Avian nephritis virus	*Mycobacterium avium*
Avian orthoreoviruses	*Mycoplasma gallisepticum*
Avian reticuloendotheliosis virus	*Mycoplasma synoviae*
Chicken anemia virus	*Salmonella pullorum*
Egg drop syndrome virus	

1.5.1 Primary Tissues

Specific pathogen-free (SPF) chicken flocks are used to supply the embryonated eggs used in vaccine manufacturing (e.g., measles, mumps, influenza, and yellow fever). The quality of the SPF flocks is controlled in the establishment, maintenance, and surveillance of the flocks (European Pharmacopoeia (Ph. Eur.), EP 5.2.2).

In the establishment of the flock, each chicken is tested repeatedly to ensure freedom from vertically transmitted agents such as leucosis viruses or antibodies that indicate a previous infection. In addition, the flock is tested to be free of a panel of selected agents (see Table 1.3) that are commonly found in conventional flocks.

The SPF flock must be maintained and monitored for two generations before certification as an SPF flock. Once formed, the flock can be used to produce new SPF flocks for egg production. All derivative flocks are maintained and routinely tested to ensure freedom from the specific agents.

1.5.2 Two-Tiered Cell Banks

Cell banking systems ensure adequate supply of well-characterized cell substrates that can last through the product life cycle or across multiple products (European Pharmacopoeia (Ph. Eur.), EP 5.2.3; FDA CBER/FDA 1993; FDA Guidance for Industry 2006, 2010). Control of manufacturing cell banks is maintained through an understanding of the history and general characteristics of the cells, cell banking systems, and quality control testing of the derivative products (ICH, Q5A(R1); ICH, Q5D). Generally, a two-tiered system is used, which consists of a Master Cell Bank (MCB) and a manufacturer's Working Cell Bank (WCB) for vaccine manufacturing (Fig. 1.3).

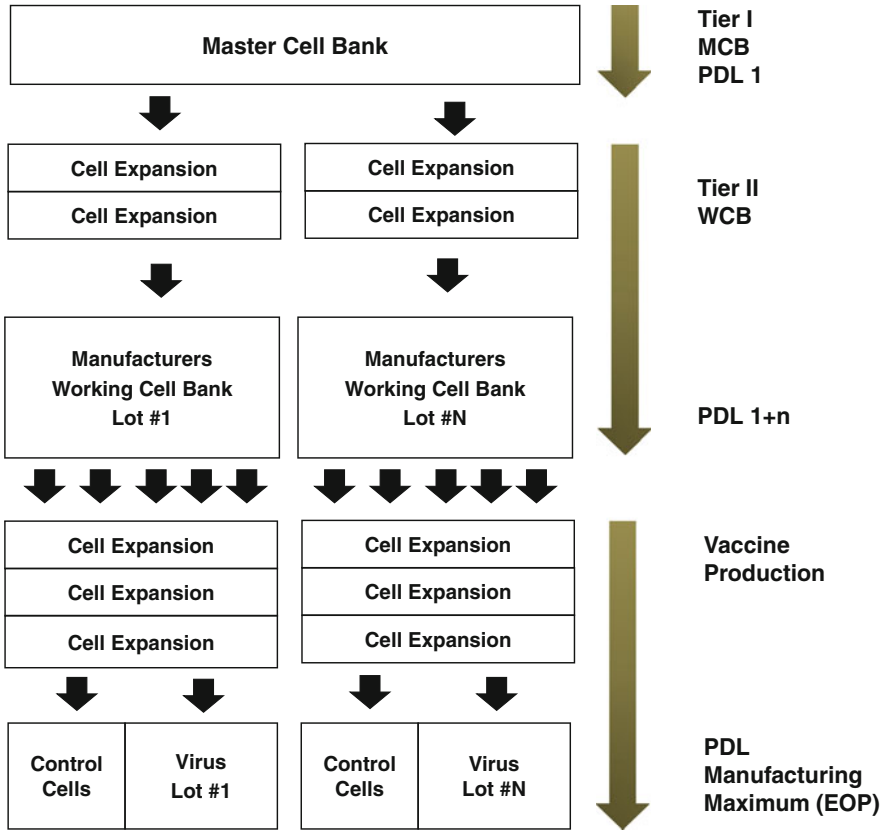


Fig. 1.3 Vaccine manufacturing using a two-tiered cell banking system

The MCB is the principal (first) defined source of cells for vaccine manufacturing and is the most proximal material for the introduction of any historically derived extraneous agents. The MCB defines the characteristics of the cell substrate (e.g., karyotype, animal tumorigenicity, clonality, morphology, productivity, absence of extraneous agents, etc.) for vaccine manufacturing, and it is the progenitor for all subsequent WCBs.

As shown in Fig. 1.3, the MCB is periodically expanded to derive a new WCB that may have coverage for several years. Each WCB, like the MCB, is further characterized using a similar panel of tests to assure the purity and safety of the cells (e.g., karyotype, animal tumorigenicity, clonality, morphology, productivity, absence of extraneous agents, etc.) for vaccine manufacturing.

The MCB is established at a specific population doubling level (PDL) that represents the number of generations (doubling) from a determined point in time. This represents the first tier (Tier I) of the cell banking system. Subsequently, the MCB is expanded to prepare one or more WCB lots at a defined PDL (Tier II).

The WCB is used to manufacture multiple lots of vaccine (control cells and bulk) at a specified maximum PDL, termed the end-of-production (EOP) cells.

For testing purposes, the MCB should be expanded to the maximum PDL at which the cell line would be used for vaccine manufacturing, termed EOP cells (European Pharmacopoeia (Ph. Eur.), EP 5.2.3; FDA Guidance for Industry 2010). The EOP cells are characterized to demonstrate the stability of cell substrate and confirm the absence of extraneous agents that might be present in the MCB. Based on the historical safety performance of human diploid cell lines (MRC-5, WI-38, FRhL-2), concern regarding tumorigenicity is not considered relevant for these cell substrates (FDA Guidance for Industry 2010; United States Pharmacopeia and The National Formulary, USP <1235>).

1.5.3 Control Cells

As shown in Fig. 1.3, the WCB is expanded and used for manufacture of each batch of the drug substance. At the time of the final cell expansion or virus infection, a number of production vessels (e.g., roller bottles or flasks) are reserved as control (uninfected) cells (European Pharmacopoeia (Ph. Eur.), EP 2.6.16. Tests for Extraneous Agents in Viral Vaccines for Human Use; United States Pharmacopeia and The National Formulary, USP <1235>). The control cells continue to be handled and maintained under conditions that are identical to those used in the vaccine virus production. However, the cells are monitored (visually and microscopically) to be free of extraneous agents (absence of cytopathic effects), they are subsequently specifically tested to be free of hemadsorbing viruses (absence of hemadsorption to the cells or hemagglutination using the culture medium), and they may be used for cell line identity testing. The culture medium used for the control cells is further evaluated in safety tests. The (direct) tests of the control cells and the culture medium are listed in Table 1.4.

It should be noted that in the manufacture of vaccines using primary cells (e.g., chick embryo cells from embryonated eggs, Sect. 1.5.1) control cells are also prepared and evaluated for the absence of extraneous agents. In both cell substrate systems, control cells function as sentinel indicators for extraneous agent(s) introduction during manufacturing of the virus stock seed lots and each bulk vaccine lot.

1.5.4 Analytical Testing Methods for Cell Substrates

Typically, the MCB and WCB are tested for extraneous adventitious agents to ensure their safety for use in vaccine production. The control cells are tested with each vaccine virus lot to further assure that no extraneous agents have been introduced during manufacturing, and to confirm the identity of the cell substrate (where multiple cell lines are used in a multi-product manufacturing site).

Table 1.4 Routine analytical methods for master cell bank, working cell bank, control cells, and end-of-production (EOP) cells

Test	MCB	WCB	Control cells	EOP
Identity	+	+	+	+
Sterility (bacterial and fungal)	+	+	+	+
Mycoplasma/Spiroplasma	+	+	+	+
Mycobacterium	+	+	–	+
Cytopathic effects (CPE) and hemadsorption/ hemagglutination	–	–	+	–
In vitro tests for adventitious agents	+	+	+	+
In vivo tests for adventitious agents	+	+	+	+
Bovine viruses (if applicable)	+	+	+	+
Porcine viruses (if applicable)	+	+	+	+
Retroviruses/retrovirus elements	+	–	–	+
Specific agents	+	+	–	–
Transmission electron microscopy	+	–	–	+
Induction of antibodies to selected agents: MAP, HAP, RAP (if applicable)	+	–	–	–
Tumorigenicity	+	–	–	+
Oncogenicity	–	–	–	+

Some of the agents and testing methods used to confirm their absence and other safety and purity tests performed on the various cell substrate intermediates are listed in Table 1.4. It should be noted that not all tests are applicable for all cell substrates, and that during the course of product life-cycle management (which can last for several decades), regulatory requirements will evolve to assure continuous improvement in product safety. Some of these analytical tests are also described in further detail in other chapters (e.g., safety testing).

The following descriptions of the routine analytical assays for MCB and WCBs illustrate both the complexity of the methods and the focus on safety testing that ensure the final quality of the vaccine product.

The requirements for these tests are described in various compendial chapters of the US Pharmacopoeia National Formulary (USP), European Pharmacopoeia (EP), British Pharmacopeia (BP), in statutory requirements of the US Code of Federal Regulations (CFR), Japanese Minimum Requirements for Biological Products (MRBP), and/or recommendations by relevant authorities (US Food and Drug Administration (FDA), European Medicines Agency (EMA), World Health Organization (WHO). Many tests are required of all live attenuated viral vaccines (e.g., identity, sterility), while others are specific to a particular vaccine product (e.g., absence of porcine agents).

1.5.4.1 Identity

Cell line identity tests are performed on cells used and generated in manufacturing. Testing provides assurance that the cell line used for vaccine manufacturing (MCB, WCB) is correct, and it is an important consideration in facilities using multiple cell lines. Testing methods include karyology, isoenzyme (e.g., lactate dehydrogenase, glucose phosphate isomerase) analyses, and nucleic acid-based technologies such as polymerase chain reaction and nucleotide sequence analysis (European Pharmacopoeia (Ph. Eur.), EP 5.2.3; FDA Guidance for Industry 2010; US Code of Federal Regulations 21 CFR § 610.18).

1.5.4.2 Sterility (Bacterial and Fungal)

Testing for the absence of microbial (bacterial and fungal) organisms is performed on cells generated and used in manufacturing. This test assures that no cultivatable microorganisms are present in the cell bank using microbial culture media and conditions (temperature, aerobic, anaerobic, length of incubation) that are compendial or accepted by regulatory authorities (European Pharmacopoeia (Ph. Eur.), EP 2.6.1; United States Pharmacopeia and The National Formulary, USP <71>; US Code of Federal Regulations 21 CFR § 610.12).

1.5.4.3 Mycoplasma/Spiroplasma

Testing for the absence of mycoplasma, a common contaminant of cell culture, is performed on cells generated and used in manufacturing. This testing method assures that no cultivatable microorganisms are present in the cell bank using direct microbial culture (e.g., in media known to be capable of cultivating *Mycoplasma* using agar and broth media) and also in an indicator cell culture using conditions (culture medium, temperature, aerobic, anaerobic, length of incubation) that are compendial or accepted by regulatory authorities (Chen 1977; European Pharmacopoeia (Ph. Eur.), EP 2.6.7; United States Pharmacopeia and The National Formulary, USP <63>; US Code of Federal Regulations 21 CFR § 610.30).

1.5.4.4 *Mycobacterium* (if Applicable)

Cell banks (MCB and WCB) and cells that have been propagated at or beyond EOP should be tested for the absence of *Mycobacteria*.

The testing method uses three different culture media (two solid media and one liquid medium) and incubation for at least 56 days under conditions that are accepted by regulatory authorities. A portion of the test article is spiked with a positive control (reference) strain of *Mycobacterium tuberculosis* to ensure validity of the test (European Pharmacopoeia (Ph. Eur.), EP 2.6.2; Siddiqi et al. 1981).

1.5.4.5 Cytopathic Effects (CPE) and Hemadsorption/ Hemagglutination

Testing is performed on control cells generated and used in manufacturing. The cells are assessed microscopically for the presence of cytopathic effects (CPE) indicating the potential for virus contamination (Albrecht et al. 1996; European Pharmacopoeia (Ph. Eur.), EP 2.6.16; FDA CBER/FDA 1993; Hotchin et al. 1958; United States Pharmacopeia and The National Formulary, USP <1237>). In addition, cell monolayers are directly tested for hemadsorption or cell lysates by hemagglutination testing to demonstrate the absence of noncytopathic viruses that contain hemagglutinin (proteins that bind erythrocytes) (Hahon et al. 1973).

While CPE may be assessed in real time during LVV manufacturing by microscopic observation, hemadsorption/hemagglutination is performed at the end of vaccine production or a minimal period of time post-infection (e.g., 14 days after addition of the vaccine virus seed).

The hemadsorption/hemagglutination assay is performed at two different temperatures (e.g., 2–8 °C and room temperature) using erythrocytes (e.g., guinea pig, chicken, human type O, or monkey), buffers, culture media, and conditions of incubation that are compendial or accepted by regulatory authorities.

1.5.4.6 In Vitro Tests for Adventitious Viruses

In vitro testing for the absence of adventitious viruses is performed on cells used and generated in manufacturing. In vitro cell safety testing may include inoculation of

- cell monolayer cultures of the same species and tissue type as that used for production of the cell bank;
- cell monolayer cultures of human diploid cells; and
- cell monolayer cultures of monkey kidney cells.

The inoculum consists of cell lysates and their culture medium, and testing conditions (length of incubation, blind passage, etc.) that are compendial or accepted by regulatory authorities are used (European Pharmacopoeia (Ph. Eur.), EP 2.6.16; FDA CBER/FDA 1993; United States Pharmacopeia and The National Formulary, USP <1237>). Evaluation consists of verifying the absence of CPE or hemadsorption (guinea pig, chicken and human or rhesus monkey erythrocytes) at specific temperatures. Unlike the in vivo testing methods, larger volumes can be tested over multiple culture vessels.

1.5.4.7 In Vivo Tests for Adventitious Viruses

In vivo testing for the absence of adventitious viruses is performed on cells used and generated in manufacturing. In vivo adventitious agent testing includes inoculation of adult and suckling mice and inoculation of embryonated chicken eggs

(allantoic and yolk sac). Additionally, in some cases, alternate animal species including guinea pigs and rabbits are used. The inoculum consists of cell lysates and their culture medium, and testing conditions (animal numbers, length of incubation, blind passage, etc.) that are compendial or accepted by regulatory authorities are used (European Pharmacopoeia (Ph. Eur.), EP 2.6.16; FDA CBER/FDA 1993; United States Pharmacopeia and The National Formulary, USP <1237>).

1.5.4.8 Bovine Viruses (if Applicable)

Testing for the absence of bovine viruses is performed on cells used and generated in manufacturing. As bovine serum (fetal or calf) is commonly used for the maintenance of cell monolayers used in vaccine manufacturing as a growth supplement, the potential introduction of bovine adventitious agents is of concern. Even if bovine serum supplemented culture media has been replaced with an alternate animal-free culture medium, previous cell exposure to bovine serum is likely to have occurred historically for the cell substrates currently approved for vaccine manufacturing (e.g., MRC-5, WI-38, Vero).

The inoculum consists of cell lysates and their culture medium, and testing conditions accepted by regulatory authorities are used. Cells used for testing include Vero and bovine (turbinate) cells. Evaluation consists of verifying the absence of CPE, testing for hemadsorption (guinea pig and chicken erythrocytes) at specific temperatures, and immunofluorescent staining for specific bovine agents (United States Pharmacopeia and The National Formulary, USP <1237>).

Currently, there is no acceptable method to test for bovine spongiform encephalopathy (BSE), a form of transmissible spongiform encephalopathy (TSE). Control to reduce the potential risk of BSE is accomplished through monitoring of source herds, geographic sourcing risk reduction, animal tissue harvesting practices, and a careful risk assessment of the vaccine manufacturing processes (European Pharmacopoeia (Ph. Eur.), EP 5.2.8).

1.5.4.9 Porcine Viruses (as Applicable)

Testing for the absence of porcine viruses is performed on cells used and generated in manufacturing. As porcine-derived trypsin is commonly used for the preparation (detachment) of cell monolayers used in vaccine manufacturing, the potential introduction of porcine adventitious agents is of concern. Even if porcine trypsin has been replaced with an alternate plant or recombinant enzyme, previous cell exposure to porcine trypsin is likely to have occurred historically for the cell substrates currently approved for vaccine manufacturing (e.g., MRC-5, WI-38, and Vero).

The inoculum consists of cell lysates and their culture medium, and testing conditions accepted by regulatory authorities are used. Cells used for testing include Vero and porcine (testes or lung) cells. Evaluation consists of verifying the absence

of CPE, testing for hemadsorption (guinea pig and chicken erythrocytes) at specific temperatures, and immunofluorescent staining for specific porcine agents (United States Pharmacopeia and The National Formulary, USP <1237>).

1.5.4.10 Retroviruses/Retrovirus Elements (if Applicable)

Retrovirus elements may need to be assessed as described in Sects. 1.5.4.11 and 1.5.4.15.

1.5.4.11 Specific Agents (if Applicable)

Testing for the absence of specific agents may be required and is performed on cells used and generated in manufacturing (European Pharmacopoeia (Ph. Eur.), EP 5.2.3; United States Pharmacopeia and The National Formulary, USP <1237>). For currently approved vaccines, this type of assessment has been used for resolving safety concerns that emerged post-marketing for chick embryo cell-derived vaccines (measles, mumps, and yellow fever) and Vero cell-derived vaccines (rotavirus).

The assessment of retroviruses (Lander and Chattopadhyay 1984; Weissmahr et al. 1997), endogenous avian viruses (EAV), and endogenous avian leucosis viruses (ALV; European Pharmacopoeia (Ph. Eur.), EP 2.6.24) in chick embryo-derived vaccines (measles, mumps, and yellow fever) was included following the detection of reverse transcriptase (RT) in the vaccine products using a highly sensitive RT assay (PCR-based enhanced RT assay or PERT) (Boni et al. 1996; Maudru and Peden 1998). While RT activity was determined to be present using PERT, no evidence was observed for ALV or EAV infection in recipients of the vaccines. The vaccines were deemed safe for continued use as their historical safety and public health benefit were deemed more significant relative to theoretical risks (Shahabuddin et al. 2001; Vesikari 2012).

The assessment of porcine circovirus type-1 (PCV-1) and type-2 (PCV-2) in Vero cell-derived vaccines (rotavirus) was performed following the detection of PCV DNA in the vaccine products using a nucleic acid technology (massive parallel sequencing, MPS), and PCR (Dubin et al. 2013; Victoria et al. 2010). While PCV DNA was determined to be present using MPS, and replication competent virus was found in one of the two major vaccines assessed (Rotarix), no evidence was observed for PCV infection in recipients of the vaccines. The vaccines were deemed safe for continued use (European Medicines Agency 2010; Kuehn 2010; World Health Organization 2010).

1.5.4.12 Transmission Electron Microscopy (TEM)

The presence of latent DNA viruses and endogenous retroviruses can be assessed by chemical induction followed by detection of induced viruses using transmission electron microscopy (TEM) and determining whether virus particles are present (European Pharmacopoeia (Ph. Eur.), EP 5.2.3; Khan et al. 2009; United States Pharmacopeia and The National Formulary, USP <1237>). It is recommended that multiple chemical inducers that have different mechanisms of action be used to demonstrate that the MCB is free of extraneous agents. While these methods are sensitive at the individual cell level, they should be coupled with other nucleic acid technologies (e.g., PCR) to improve the specificity and sensitivity to identify specific agents (Khan et al. 2009).

1.5.4.13 Induction of Antibodies to Selected Agents: MAP, HAP, RAP (if Applicable)

Mouse, hamster, and rat antibody production (MAP, HAP, RAP) testing is an in vivo method for inducing an immunological response to potential extraneous murine viral contaminants that might be present in the MCB (ICH, Q5A(R1) 1997; ICH, Q5D 1997; United States Pharmacopeia and The National Formulary, USP <1237>). This assay is performed on MCB where there is concern that a murine contaminant may be present.

Animals are inoculated multiple times with MCB cell lysates to induce antiviral antibodies, and serum is harvested after four weeks. The serum is then assessed using appropriate immunoassay(s) and conditions accepted by regulatory authorities to determine whether an immune response to targeted (extraneous) agents occurred.

1.5.4.14 Tumorigenicity

Prior to 2000, only cells that were shown to be nontumorigenic were used for the manufacture of live attenuated virus vaccines. Currently, diploid cells (MRC-5, WI-38, and FRhL-2) are considered well-characterized and nontumorigenic (FDA CBER/FDA 1993; FDA Guidance for Industry 2006; US Code of Federal Regulations 21 CFR § 610.18); however, Vero cells and new novel cell lines (e.g., MDCK cells, modified Vero cells) must be assessed for their tumorigenic potential (Onions et al. 2010). Since live attenuated virus vaccine drug products are not rigorously purified, the potential that intact cells, derived from the manufacturing substrate, could be inoculated into a vaccine recipient is of concern.

While in vitro tests for tumorigenicity (e.g., colony formation in soft agar gels, invasive cell growth in organ culture, or transformation activity for active oncogenes) may be acceptable to regulatory authorities, the preferred (gold standard) testing method consists of in vivo testing of cells used and generated in

manufacturing that have been propagated at or beyond the maximum PDL intended for use in manufacturing, referred to as the EOP (European Pharmacopoeia (Ph. Eur.), EP 5.2.3; FDA Guidance for Industry 2010).

Suitable animal systems include athymic (nude) mice and nude rats. Newborn mice, newborn rats, or newborn hamsters that have been thymectomized or treated with anti-thymocyte immunoglobulin, or irradiated mice that have been reconstituted with bone marrow from healthy mice can be used. Typically, tumor incidence is assessed using 10^7, 10^5, 10^3, and 10^1 cells per animal as the inoculum (injected intramuscularly or subcutaneously). A positive (reference) tumorigenic cell control such as HeLa or HEp-2 cells is required. At least 10 animals per group are observed for tumor incidence (i.e., tumor frequency and deaths) for up to 12 weeks (with a 3 week intermediate assessment of 50 % of the animals).

1.5.4.15 Oncogenicity (if Applicable)

As noted in Sect. 1.5.2, diploid cells (MRC-5, WI-38, and FRhL-2) are considered well-characterized and nontumorigenic (European Pharmacopoeia (Ph. Eur.), EP 5.2.3; United States Pharmacopeia and The National Formulary, USP <1237>). As nontumorigenic, these cells would also be considered free of oncogenic components (e.g., active retroviral agents or oncogenes) that could contaminate the vaccine product.

Vero cells and other new novel cell lines (e.g., PER.C6, MDCK cells, modified Vero cells) that have been immortalized (grow continuously in cell culture without senescence) must be assessed for their oncogenic potential in vivo (EP 5.2.3; United States Pharmacopeia and The National Formulary, USP <1237>). Since live attenuated virus vaccine drug products are not rigorously purified, the potential that cellular components (specifically DNA and protein), derived from the manufacturing substrate could be inoculated into a vaccine recipient is of concern. In particular is the risk that oncogenic transformation might occur following integration of a dominant oncogene or introduction of DNA that could be infectious or generate an infectious agent (e.g., retroviral DNA).

There are no validated methods for oncogenicity testing of cells that are accepted by the regulatory agencies; however, it is required that evaluation of the oncogenicity of host cell DNA and cell lysates be completed in vivo. The design of the testing method includes use of purified cellular DNA from cells representative of the cells used and generated in manufacturing that have been propagated at or beyond EOP (see Sect. 1.5.2).

The oncogenic and infectious risk of DNA is primarily addressed by lowering the amount of DNA, decreasing the size of the DNA (by digestion with nucleases, e.g., benzonase), and/or by reducing the activity of the DNA (by chemical treatment or gamma irradiation).

1.6 Biologics CMC: Manufacturing Virus Seeds

The vaccine virus seed lot system, like the cell banking system, ensures adequate supply of well-characterized virus seeds that can last through the product life cycle or across multiple products. Generally, a two-tiered seed lot system is used, which consists of a Master Virus Seed (MVS) and a manufacturer's Working Virus Seed (WVS) as shown in Fig. 1.4. The MVS is periodically expanded to derive a new WVS that may have coverage for several years. The WVS is used for manufacture of each batch of the drug substance. For products which do not use two-tiered seed lot systems, the MVS is used for manufacture of each batch of the drug substance.

Generally, virus seeds are not purified significantly, as they are starting materials for manufacturing new WVS seeds (MVS to make WVS) or bulk vaccine harvests (WVS to make drug substance). The MVS and WVS may consist of infected cell lysates, tissue culture supernatants, or allantoic fluids.

The MVS is prepared from a progenitor pre-seed that may represent a clinical isolate that has been passaged for attenuation or a reassortant virus and represents

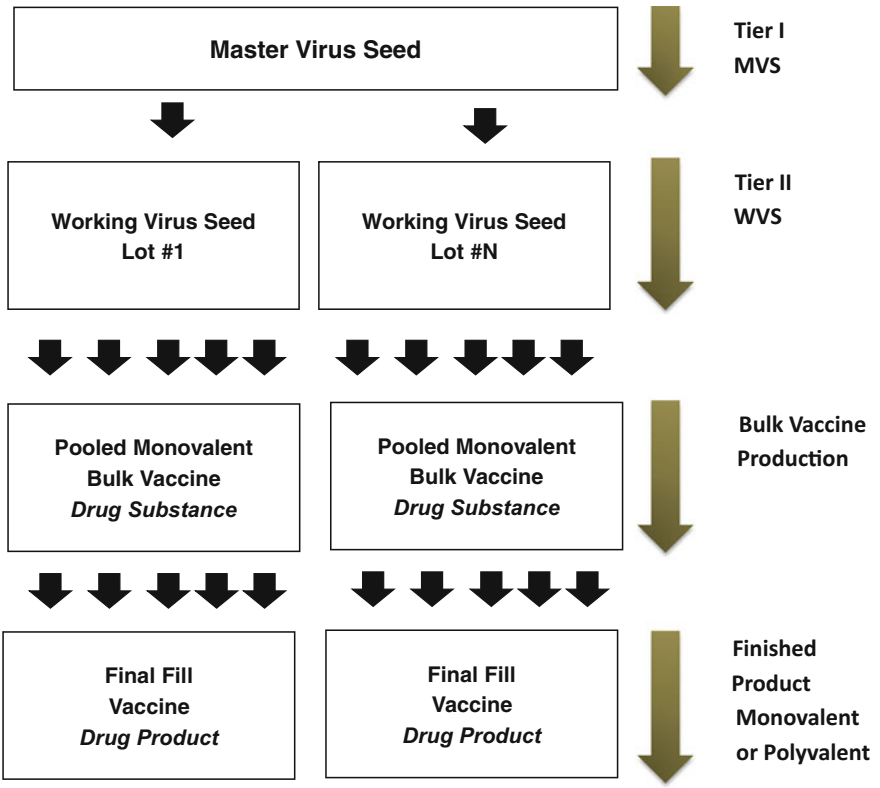


Fig. 1.4 Vaccine manufacturing using a two-tiered seed lot system

the first tier of the seed lot system. The MVS is used to prepare one or more WVS lots at a defined passage level. The WVS is then used to manufacture multiple lots of monovalent vaccine bulk at a specified maximum virus passage level. The monovalent bulk represents single or multiple collections termed harvested virus fluids (HVF).

The MVS is extensively characterized. Because this virus seed is the most proximal to the introduction of any historically derived extraneous agents, it defines the characteristics of vaccine product (e.g., genotype, phenotype, attenuation, antigenic properties, etc.). It is also the progenitor for all subsequent WVSs. In addition, the MVS may be passaged beyond the level that would be used for vaccine manufacturing and evaluated to demonstrate genetic/phenotypic stability.

Typically, MVSs and WVSs are fully characterized focusing on safety, absence of extraneous adventitious agents, and biological stability (genetic and phenotypic) (European Pharmacopoeia (Ph. Eur.), EP 5.2.3; United States Pharmacopeia and The National Formulary, USP <1235>). Extraneous agents may have been introduced during: (1) virus isolation that might have been present in the original clinical isolate; (2) successive passaging for attenuation (e.g., introduced by the cell substrate or raw materials used for virus growth); or (3) during virus rescue of attenuated reassortants [whether naturally (rotavirus) or recombinantly (influenza)].

Many of the tests (purity and safety) are identical to those performed on the cell banks. However, because these materials contain infectious virus, specific high potency antivirus (seed) antiserum is required to completely neutralize the seed virus from replicating during some safety testing, as some of the methods use permissive substrates (e.g., in vitro cell culturing, animal testing) that would confound the interpretation of the result should unneutralized seed virus replicate.

In addition, the antiserum used in these assays must be derived from virus propagated in a substrate that differs from the substrate used in vaccine manufacturing (European Pharmacopoeia (Ph. Eur.), EP 2.6.16; FDA Guidance for Industry 2010). This is to ensure that neutralization of virus replication is due to virus-specific antibodies, and not antibodies that might be derived from the substrate used for virus preparation.

The manufacturing processes for the MVS and WVS are similar to the process used for drug substance manufacturing. Generally, they are starting materials that will be used for the entire product life cycle, which may last decades, and accordingly, they are characterized more extensively for safety and absence of extraneous agents.

1.7 Biologics CMC: Drug Substance

Vaccine manufacturing occurs in a complex biological substrate with minimal downstream processing to prepare the drug substance. The quality attributes that are required for the product are in part controlled using well-characterized cell substrates and virus stock seeds, raw materials with minimal or no animal-derived

products, manufacturing consistency and controls, and extensive safety testing of the drug substance.

Cell culture preparation and expansion requires the seeding of cell suspensions into appropriate culturing vessels/bioreactors. The cells may be harvested from primary tissues (e.g., chick embryos), or they may be cryopreserved cells from a WCB. The cells are allowed to grow to some degree of confluency and are used either directly in vaccine manufacturing, or detached and expanded through one or more expansion steps to scale up the substrate to a suitable manufacturing level.

When the cell substrate is at manufacturing scale, the cells are infected with a WVS under conditions that allow the vaccine virus to replicate to levels suitable for harvesting. At a specified time post-infection (usually days), the virus is harvested from the cell culture vessels/bioreactors. In some instances, the harvested material represents infected biological fluids (e.g., allantoic fluids from embryonated eggs, or culture medium from bioreactors) and/or cell substrates. If whole cells are harvested, they may subsequently be disrupted to release the vaccine virus. Regardless of their source, these fluids are referred to as bulk HVF. The harvests may be pooled, clarified to remove intact cells (e.g., through filtration or centrifugation), and frozen. Alternatively, straight-through processing (STP) of the pooled HVF into downstream purification steps may occur.

When the cell substrate is at manufacturing scale during the manufacturing of a vaccine drug substance lot, a portion of the lot is segregated and maintained as uninfected control cells (see Sect. 1.5.3). The control cells are cultured in parallel with the vaccine production vessels (e.g., fed with the same medium, rinsed with the same buffers) and are harvested similarly to the production vessels. They are monitored through production for the absence of any cytopathogenic effects, and at the end of manufacturing, the control cells are processed and tested in parallel with the drug substance for the absence of extraneous agents (see Sect. 1.5.4).

If the HVF intermediates are stored frozen, they may subsequently be pooled with additional harvests from the same batch that are harvested later in a multi-harvest product. The HVF are generally sampled for potency testing and for critical safety assays.

Pooling and purification may be simple (i.e., no significant downstream processing other than clarification and aseptic processing) or complex (e.g., ultracentrifugation, detergent solubilization, sonication, Benzonase® treatment, tangential flow filtration (TFF)). The resulting material, which may be sterile filtered or not (with aseptic processing), becomes the vaccine drug substance. It should be noted that regardless of the complexity of the pooling and purification, an essential step is the removal of any intact viable cells by clarification and/or other process steps. The absence of intact viable cells is generally determined using microscopic observation of appropriate samples at the HVF or pooling steps of drug substance manufacturing.

Manufacturing process residuals and impurities are monitored in the drug substance (or drug product) to assure that the levels received by the end recipient will meet regulated specifications (United States Pharmacopeia and The National Formulary, USP <1235>). As viral vaccine drug substances generally have high vaccine virus potency and subsequent dilution to the drug product potency may be

significant, measurement of process residuals is best obtained from testing of the drug substance rather than the final container (drug product).

The drug substance is low stored at temperatures (e.g., < −60 °C) in containers that ensure its stability for months to years and monitored to demonstrate that it will maintain the required quality attributes so that it can be diluted appropriately into the drug product.

1.8 Biologics CMC: Drug Product

Preparation of the vaccine drug product uses a final formulated bulk which can consist of a monovalent drug substance (e.g., monovalent varicella vaccine), mixtures of drug substances manufactured from different virus serotypes/subtypes (e.g., adenovirus, rotavirus, and influenza vaccines), or mixtures (combinations) of drug substances manufactured from different viruses (e.g., measles, mumps, rubella, and varicella into a quadrivalent vaccine). The final formulated bulk is prepared with stabilizing excipients and/or buffers, and it is subsequently filled into the final container (e.g., vial, syringe, sprayer, oral dispensing tube). While most live virus vaccines products are lyophilized (e.g., in vials), some products may be distributed as a liquid product (e.g., influenza in sprayers, rotavirus in dispensing tubes).

As the drug products are generally administered parenterally, sterility of the drug product and container closure integrity throughout the product shelf life are essential quality standards. After filling, the final containers are labeled and stored until tertiary packaging may occur. Tertiary packaging includes assembly of the final container with the package insert and packaging into trays, boxes, or cartons.

Since LVV products require virus replication in order to induce immunity, the potency assay for an LVV provides a surrogate to the vaccine's mechanism of action in humans. Figure 1.5 shows a model for understanding the relationship between the drug product release specifications and the dose claim. The Minimum Release Potency of the drug product is sufficient to allow for loss of virus potency across the manufacturing process, packaging and transport, during storage pre-distribution and post-distribution, and following reconstitution or handling at ambient temperature. This ensures that the dose claimed for inducing immunity (dose claim) is maintained across the product shelf life (e.g., 2 years at 2–8 °C for measles, mumps, rubella vaccines).

In Fig. 1.5, the *y*-axis represents potency and the *x*-axis is time. Provided that the drug substance potency is sufficient to manufacture the drug product, the drug substance is diluted to a target potency that accounts for process loss, process variability, and assay variability. This value minimizes the risk that the product does not meet a minimum release specification or exceed a maximum release specification.

The upper safety specification represents the measured potency for the *highest* dose (determined with a clinical lot) which was found to be safe in a clinical study. This may be higher than the maximum release specification as shown in Fig. 1.5, or alternatively it could be identical to the maximum release specification.

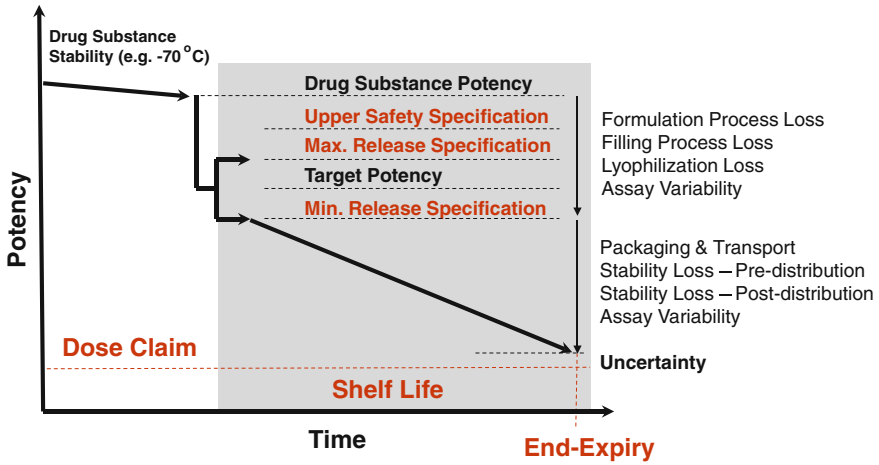


Fig. 1.5 Drug product potency specification and stability model

To determine the minimum release potency, specification three pieces of information are needed: (1) the dose claim (minimum effective dose); (2) the final container stability data across the product shelf life (shaded area in Fig. 1.5); and (3) an assay variability (uncertainty) term. The dose claim is the measured potency for the *lowest* dose found to be efficacious in a clinical study. The dose claim is not the minimum release specification, since live virus vaccines tend to lose potency over their shelf life.

To establish the minimum release specification, the end expiry dating accounts for the variability of the potency assay (uncertainty) and the shelf life potency decay slope. The stability loss is derived from stability data measured on typical pre-licensure lots during development.

Figure 1.5 illustrates potency (*y*-axis) loss over time (*x*-axis) for a model vaccine drug product due to the manufacturing process and post-production conditions. The potency specifications ensure that the delivered dose (to the vaccinee) meets the dose claim over the intended shelf life (shaded area) of the vaccine product.

1.9 Biologics CMC: Analytical Testing Methods of Seeds, Intermediates, Drug Substance, and Drug Product

1.9.1 Analytical Considerations for Virus-Containing Materials

Typically, MVS, WVS, HVF, drug substance, and drug product analytical methods are focused on safety and absence of extraneous adventitious agents (European Pharmacopoeia (Ph. Eur.), EP 5.2.3, United States Pharmacopeia and The National

Formulary, USP <1235>). Many of the purity and safety tests are identical to those performed on the cell banks. However, because these materials contain infectious virus, specific high potency antivaccine virus antiserum is required to completely neutralize the virus component from replicating during some safety testing, as some of the methods use permissive substrates (e.g., in vitro cell culturing, in vivo animal testing) that would confound the interpretation of the result should the drug substance vaccine virus replicate.

In addition, the antiserum used in these assays must be derived from virus propagated in a substrate that differs from the substrate used in vaccine manufacturing. This is to ensure that neutralization of virus replication is due to virus-specific antibodies, and not antibodies that might be derived from the substrate used for virus preparation (European Pharmacopoeia (Ph. Eur.), EP 2.6.16; FDA Guidance for Industry 2010).

Another consideration for sterility testing is associated with manufacturing processes that use antibiotics as a supplement to the culture medium (e.g., neomycin, gentamicin). In these cases, testing for microbial (bacterial and fungal) organisms requires filtration and testing of the filter for retained cultivatable organisms.

1.9.2 Analytical Methods

The routine analytical methods used for testing virus-containing products and intermediates (MVS, WVS, HVF, drug substance, and drug product) are listed in Table 1.5. The requirements for these tests are described in various compendial chapters of the US Pharmacopoeia National Formulary (USP), European Pharmacopoeia (EP), British Pharmacopeia (BP), in statutory requirements of the US Code of Federal Regulations (CFR), Japanese Minimum Requirements for Biological Products (MRBP), and/or recommendations by relevant authorities (US Food and Drug Administration (FDA), European Medicines Agency (EMA), World Health Organization (WHO)). Many tests are required of all live attenuated viral vaccines (e.g., identity, sterility), while others are specific to a particular vaccine product (e.g., absence of porcine agents). Some of these analytical tests are also described in further detail in other chapters (e.g., safety testing).

1.9.2.1 Potency

All live viral vaccines use infectivity assays to measure potency. The method may be quantal (median cell culture infectious dose, CCID50, median tissue culture infectious dose, TCID50, median egg infectious dose, EID50) or quantitative (plaque forming assay, fluorescence focus assay, quantitative polymerase chain reaction). These assays are cell-based assays, which measure the replication (TCID50 and Plaque Assay) or infectivity (FFA) of the vaccine virus component.

Table 1.5 Typical analytical methods for many master virus seed (MVS), working virus seed (WVS), harvested virus fluids (intermediate), drug substance, and drug product

Tests	MVS	WVS	HVF	Drug substance	Drug product
Vaccine virus					
Potency/infectivity	+	+	+	+	+
Identity	+	+	–	+	+
Thermal stability	–	–	–	–	+
Attenuation (influenza)	+	+	–	+	–
Genotype (influenza, poliovirus)	+	+	–	+	–
Phenotype (influenza)	+	+	–	+	–
Neurovirulence	+	+	–	–[a]	–
Total Particle (qPCR, TEM)[b]	+	+	–	+	–
Purity and safety					
Sterility (bacterial and fungal)	+	+	+	+	+
Mycoplasma/Spiroplasma	+	+	–	+	–
In vitro tests for adventitious agents	+	+	–	+	–
In vivo tests for adventitious agents	+	+	–	+	–
Intact cell assay	–	–	–	+	–
Bioburden (if applicable)	+	+	+	–	–
Mycobacterium (if applicable)	+	+	–	+	–
Bovine viruses (if applicable)[c]	+	+	–	+	–
Porcine viruses (if applicable)[c]	+	+	–	+	–
Specific agents (if applicable)[c]	+	+	–	+	–
RT assay (if applicable)	+	+	–	+	–
Manufacturing process residuals[d]					
Bovine serum (if applicable)	–	–	–	+	–
Egg ovalbumin (if applicable)	–	–	–	+	–
Antibiotics (neomycin, gentamicin)	–	–	–	+	–
Total protein (yellow fever, smallpox)	–	–	–	+	–
Residual benzonase (influenza, rotavirus)	–	–	–	+	–
Residual host cell DNA[b]	–	–	–	+	–
Residual host cell protein[b]	–	–	–	+	–
Product specific					
Color and/or appearance	–	–	–	+	+
Endotoxin	–	–	–	–	+
General safety testing	–	–	–	–	+
Osmolality	–	–	–	–	+

(continued)

Table 1.5 (continued)

Tests	MVS	WVS	HVF	Drug substance	Drug product
pH	–	–	–	–	+
Moisture content (lyophilized products)	–	–	–	–	+

[a] Limited number (e.g., four) of drug substance consistency lots (first lots manufactured to demonstrate manufacturing process control and consistency). Monovalent poliovirus bulks are tested for neurovirulence

[b] Assay is used for characterization (c) in support of licensure

[c] Testing for the absence of these agents may use the control cells (see Sect. 1.5.3) rather than the drug substance

[d] Most process residuals are present at very low quantities that make assays impractical to measure them in the final product due to dilution of the drug substance. However, the specifications are implemented on the final product dose

For combination and polyvalent drug products, further complexity is introduced in the analytical methods. For potency assays, neutralization of all virus components, except the virus component being measured may be required. Thus, measurement of the measles virus potency in the trivalent measles, mumps, and rubella vaccine requires neutralization of the mumps and rubella components. For some products (e.g., influenza and rotavirus), neutralization is not required, and detection of each component is determined using virus-specific analytical reagents (e.g., virus-specific immunostaining for influenza, polymerase chain reaction (PCR) amplification with primer-probes designed for serotype specific analysis for rotavirus).

Potency: CCID50, TCID50, EID50, and Mouse LD50 Assay

In quantal assays, infectivity (adenovirus, poliovirus, measles, mumps, rubella, rotavirus, yellow fever virus) is estimated by infecting multiple units of a permissive substrate (e.g., 3–8 eggs or 6–12 cell culture wells) with the test inoculum. The test inoculum is serially diluted (e.g., four-, five-, or tenfold), and each dilution is used to infect an identical number of substrate units. The infectivity titer is then calculated based on the amount of inoculum that is required to infect 50 % of the substrate units (e.g., 4/8 eggs or 4/8 cell culture wells) (Daelemans et al. 2011; LaBarre and Lowy 2001; Umino et al. 1990; Yeolekar and Dhere 2012).

For cell culture assays, infectivity is measured by direct microscopic observations of CPE that result from virus replication, or by using a secondary staining of cells (e.g., a vital stain to determine infected wells or an immunostain to detect virus antigen expression). For substrates such as eggs, the allantoic fluids are tested in a hemagglutination (HA) assay to determine virus replication.

Yellow fever vaccine was originally licensed using a median mouse lethal dose (mouse LD50). That represents the quantity of virus that will produce fatal encephalitis in 50 % of susceptible mouse strains following intracerebral inoculation (Theiler and Smith 1937). While this assay is still relevant, potency is now determined using a plaque assay that has been validated to give the equivalent infectious potency as the mouse LD50 (European Pharmacopoeia (Ph. Eur.) EP monograph 01/2013:0537).

Potency: Plaque Assay

The plaque assay (smallpox, varicella, yellow fever) is a quantitative cell-based infectivity assay that measures virus particle replication and spread (plaque formation). Typically, the test inoculum is serially diluted (e.g., four-, five-, or tenfold) and each dilution is used to infect multiple (e.g., 2–4) replicate wells/dishes of the permissive cell substrate. After an appropriate length of incubation (e.g., 6–7 days post-infection), the cell monolayers are stained, plaques are counted, and the virus titers are calculated based on plaque number and dilution (Dulbecco and Vogt 1954).

Potency: Fluorescent Focus Assay (FFA)

The FFA assay (influenza) is a quantitative cell-based infectivity assay that measures influenza virus particle antigen expression. Typically, the test inoculum is serially diluted (e.g., four-, five-, or tenfold) and each dilution is used to infect multiple (e.g., 2–4) replicate wells/dishes of the permissive cell substrate. After an appropriate length of incubation (e.g., 24-h post-infection), immunostaining (surface glycoproteins HA and NA) is used to identify and enumerate infected cells, which are counted. The virus titers are calculated based on foci number and dilution (Yang et al. 1998).

Potency: Multivalent Quantitative RT-PCR Assay (M-QPA)

The M-QPA assay (rotavirus, RotaTeq®) is a quantitative cell-based infectivity assay that measures rotavirus replication in permissive cells using quantitative RT-PCR to measure viral RNA relative to a rotavirus reference standard (Ranheim et al. 2005).

In the assay, the test inoculum and pentavalent rotavirus reference standard are serially diluted and each dilution is used to infect multiple replicate wells of the permissive (Vero) cell substrate. After an appropriate length of incubation (e.g., 24-h post-infection), the cells are lysed, and viral RNAs are quantified by RT-QPCR. The primers and probes used for amplification allow specific quantitation of each serotype virus in the presence of the other four viruses (Ranheim et al. 2005).

1.9.2.2 Identity

To assure the identity of the vaccine virus, assays are performed that use specific antidrug substance antiserum to confirm virus identity (United States Pharmacopeia and The National Formulary, USP <1235>; US Code of Federal Regulations 21 CFR § 610.14). The identity test generally uses virus neutralization or alternatively immunostaining with specific antisera. The antiserum used in this assay must be derived from virus propagated in a substrate that differs from the substrate used in vaccine manufacturing. In addition, for high potency (titer) materials (e.g., drug substance), high potency antiserum is required to ensure viral neutralization.

For products that may contain multiple serotypes (e.g., influenza, rotavirus), molecular genotyping may be used.

1.9.2.3 Thermal Stability

For some LVV products, thermal stability after incubation at a temperature higher than is recommended for storage is completed (United States Pharmacopeia and The National Formulary, USP <1235>). The duration of incubation (e.g., hours/days) and temperature of incubation (e.g., 37 °C) reflect potential real-life excursions that might impact the quality of the product (Brandau et al., 2003). Typically, drug product potency loss is the measured attribute (e.g., no greater than 0.5 log10 CCID50 loss for poliovirus after incubation at 37 °C for 48 h). The potency loss is relative to containers stored at the temperature recommended for product storage (e.g., for poliovirus vaccine, 2–8 °C).

1.9.2.4 Attenuation

To assure the maintenance of attenuation, in vivo testing may be performed (e.g., for influenza, poliovirus, yellow fever virus, and smallpox vaccines) to assure that the master virus seed and drug substance is sufficiently attenuated.

Using influenza as an example, in vivo testing is completed to demonstrate that virus replication in the lungs is reduced in ferrets. Healthy nonimmune ferrets are inoculated intranasally and monitored. The dose (potency and volume) used for inoculation is specific for each virus, and the animals must be observed for clinical symptoms for specific times post-inoculation (e.g., 3 days). The nasal turbinates and lungs are harvested at the end of the observation period and homogenized. The homogenates are then assessed in embryonated eggs for detectable virus titers using an EID50 assay (see Sect. 1.9.2.1). The virus drug substance is attenuated if replication occurs in the nasal turbinates and no detectable replication occurs in the lungs (Belser et al. 2011; Jin et al. 2003, 2004).

1.9.2.5 Genotype

Since the genetic sequence defines the phenotypic properties of the drug substance vaccine virus, the stability of the vaccine virus genome is a measure of the maintenance of attenuation. Each MVS and WVS may be sequenced in its entirety and then compared with virus propagated in EOP cells (see Sect. 1.5.2). This approach is limited as it deduces the vaccine virus genome population consensus nucleotide sequence, and does not fully measure population mixtures (e.g., genotype mixtures) or dispersity (e.g., genomic quasi-species) (Poon et al. 2010).

More typically, genotype analysis is restricted to a subset of the genome as it can be applied to the MVS, WVS, and individual vaccine bulk harvests (drug substance precursors). In this application, targeted amplicons (e.g., attenuation loci, or sequence markers for differentiation) from the virus genome are amplified (e.g., RT-PCR) and then further analyzed by size and restriction fragment polymorphisms (RFP). This analysis can be quantal, to demonstrate the maintenance and clonality of the drug substance (e.g., influenza master donor virus segments) or quantitative to demonstrate the relative abundance of genotypes (e.g., mutant analysis by PCR and restriction enzyme cleavage, MAPREC, for poliovirus) (European Pharmacopoeia (Ph. Eur.), EP monograph 01/2012:0215).

1.9.2.6 Phenotype Stability

Phenotype characteristics of a LVV drug substance are confirmed by phenotype assays specific for a particular LVV drug substance. For example, phenotype testing is completed to demonstrate maintenance of cold adaptation (ca) and temperature sensitivity (ts) of the influenza vaccine virus. These attributes are a measure of virus growth (at 25 °C, ca) or growth restriction (at 37 °C for B types or 39 °C for A types, ts) compared to the growth observed at 33 °C. The ts restriction is expected to reduce virus replication by at least 200-fold (2 log10) at the elevated temperature; whereas ca allows the virus to grow at the reduced temperature with no less than 200-fold (2 log10) difference from the growth observed at 33 °C (Chen et al. 2006; Hoffmann et al. 2005; Jin et al. 2003).

1.9.2.7 Neurovirulence

Each MVS and WVS may be required to comply with monkey neurovirulence testing requirements (European Pharmacopoeia (Ph. Eur.), EP 2.6.18; FDA Guidance for Industry 2010). The purpose of this method is to demonstrate the absence of neurovirulent properties of the seeds. Historically, nonhuman primates were used, and only recently have other animal models (e.g., rodents) been assessed as potential replacement models (Rubin 2011; Afzal et al. 1999).

Healthy nonimmune monkeys that are susceptible to the specific virus (e.g., Macaca species for yellow fever virus) are inoculated by intracerebral and

intraspinal routes. The dose (potency and volume) used for inoculation is specific for each virus, and the animals must be observed for specific times post-inoculation (e.g., 30 days).

Assessment may include demonstration of viremia (detectable virus in blood to specific levels) and immunogenicity (assessed by neutralization titers to API) to assure virus replication has occurred. Neurotropism is assessed by clinical and histopathological analysis. For most live attenuated viral vaccines, the MVS and WVS should demonstrate no unexpected clinical or histopathological evidence attributable to the inoculated virus.

For poliovirus and yellow fever virus, comparison to a reference vaccine strain is performed, as there is expected histopathological evidence for virus replication (Levenbook et al. 1987). For these viruses, lesion scores must be comparable to those of the reference vaccine. For poliovirus, this test (or an alternate test using a transgenic mouse model) is also required for each monovalent pooled harvest.

1.9.2.8 Total Particles (Characterization)

Characterization of the virus component may include particle numbers and morphology. This is accomplished by direct observation of particles (e.g., cryo electron microscopy or TEM) or quantitative measurements of genome copy numbers using nucleic acid technologies (qPCR, van Elden et al. 2001).

1.9.2.9 Sterility (Bacterial and Fungal)

This test assures that no cultivatable microorganisms are present using microbial culture media and conditions (temperature, aerobic, anaerobic, length of incubation) that are compendial or accepted by regulatory authorities (European Pharmacopoeia (Ph. Eur.), EP 2.6.1; United States Pharmacopeia and The National Formulary, USP <71>; US Code of Federal Regulations 21 CFR § 610.12).

1.9.2.10 Mycoplasma/Spiroplasma

For method description, refer to Sect. 1.5.4.3.

1.9.2.11 In Vitro Adventitious Agent Testing

For method description, refer to Sect. 1.5.4.6.

1.9.2.12 In Vivo Adventitious Agent Testing

For method description, refer to Sect. 1.5.4.7.

1.9.2.13 Intact Cells

Following clarification of the drug substance, a sample is assessed microscopically to determine the absence of viable intact cells using a vital stain (United States Pharmacopeia and The National Formulary, USP <1235>).

1.9.2.14 Bioburden (if Applicable)

For products manufactured in embryonated eggs (e.g., influenza), there is an unavoidable introduction of microorganisms into harvested allantoic fluids. The bioburden test is performed as part of in-process testing on samples of the HVF and drug substance prior to sterilizing filtration.

The bioburden test is used to detect and quantify the microbial population of aqueous in-process samples. All morphologically distinct organisms are isolated and Gram-stained. The bioburden test method is performed using microbial culture media and conditions (temperature, aerobic, anaerobic, length of incubation) that are compendial or accepted by regulatory authorities (United States Pharmacopeia and The National Formulary, USP <61>; United States Pharmacopeia and The National Formulary, USP <62>).

1.9.2.15 Mycobacteria

For method description, refer to Sect. 1.5.4.4.

1.9.2.16 Bovine Viruses (if Applicable)

For method description, refer to Sect. 1.5.4.8.

1.9.2.17 Porcine Viruses (if Applicable)

For method description, refer to Sect. 1.5.4.9.

1.9.2.18 Specific Agents (if Applicable)

For method description, refer to Sect. 1.5.4.11.

1.9.2.19 RT Assay (if Applicable)

For method description, refer to Sect. 1.5.4.10.

1.9.2.20 Bovine Serum

A number of vaccine manufacturing processes use bovine serum (fetal or calf) as a growth supplement during cell substrate expansion. However, the serum is removed and the cell substrate is rinsed before harvesting of the virus fluids. In order to determine the level of residual serum present, bovine serum albumin is measured in the drug substance as a surrogate for bovine serum. While the typical requirement of not more than 50 ng per single human dose is applied to the drug product, because of the low levels of residual bovine albumin, testing is completed on the drug substance. Testing is performed using a suitable immunochemical method (European Pharmacopoeia (Ph. Eur.), EP 2.7.1; United States Pharmacopeia and The National Formulary, USP <1235>).

1.9.2.21 Egg Ovalbumin (Specific for Influenza)

For embryonated egg-based products, residual ovalbumin in the drug product is of concern. While the typical requirement to limit the amount of ovalbumin per single human dose is applied to the drug product, because of the low levels of residual ovalbumin, testing is completed on the drug substance. Testing is performed using a suitable immunochemical method under conditions that are compendial or accepted by regulatory authorities (European Pharmacopoeia (Ph. Eur.), EP 2.7.1).

1.9.2.22 Antibiotics

For products that use primary cells (e.g., chick embryo cells) that produce vaccine virus, as well as for products that use established cell lines to produce vaccine virus, antibiotics (e.g., neomycin, gentamicin) may be used in manufacturing of the MVS, WVS, and HVF and/or in final formulation. Depending on the manufacturing process, antibiotics may be present in the finished drug product in trace amounts or at higher levels. The potency of the antibiotic may be measured in two ways. The cylinder plate assay depends on diffusion of the antibiotic from a vertical cylinder through solidified agar in a Petri dish. The antibiotic prevents growth of specific organisms inoculated into the agar in a circular area around the antibiotic-containing cylinder. The turbidimetric assay depends on inhibition of the growth of a microorganism when the antibiotic is included in a fluid medium in which the microorganism would otherwise grow (European Pharmacopoeia (Ph. Eur.), EP 2.7.2; United States Pharmacopeia and The National Formulary, USP <81>).

1.9.2.23 Total Protein (if Applicable)

Total protein in LVV drug product (e.g., yellow fever virus vaccine) is determined by routine protein assays (European Pharmacopoeia (Ph. Eur.), EP 2.2.33; United States Pharmacopeia and The National Formulary, USP <1057>). The assay methods are compendial or accepted by regulatory authorities (USP-NF, EP).

1.9.2.24 Residual Benzonase®

In some products, host cell DNA present in the drug substance is fragmented by Benzonase®, a nonspecific endonuclease, to reduce and eliminate the amount of DNA present in the final product. The enzyme is subsequently removed by ultrafiltration.

The residual Benzonase® present in the drug substance is determined using a Benzonase® enzymatic activity assay by its ability to cleave herring sperm DNA. The live vaccine drug substance test article is compared against a Benzonase® standard curve, in which readings are measured with a spectrophotometer at 260 nm (Sheng-Fowler et al. 2009).

1.9.2.25 Residual Host Cell DNA (Characterization)

For vaccines manufactured in primary cells or diploid cells, the presence of residual host cell DNA (hcDNA) is not a regulatory or safety concern. However, for products manufactured in Vero cells (e.g., rotavirus, smallpox) or other continuous cell (hcDNA) lines (e.g., MDCK), the presence of residual hcDNA is of concern (Sheng-Fowler et al. 2009).

Residual hcDNA is quantified using nucleic acid technology methods (e.g., real-time PCR). In general, high copy number sequences (e.g., highly repetitive genomic DNA satellite sequences) from the cell substrate are amplified in the assay. The cell-specific amplification product is generated from the residual hcDNA template and compared with reference samples using a set of optimized oligonucleotide primers (Nissom 2007).

1.9.2.26 Residual Host Cell Protein (Characterization)

The amount of residual host cell protein (HCP) in the drug substance may need to be determined. Generally, an immunological test method (enzyme-linked immunosorbent assay, ELISA) is used to assess the drug substance. As an example, the ELISA may use a capture anti-HCP antibody (e.g., immune rabbit antiserum to HCP) as the immunosorbent, a primary biotin labeled anti-HCP antibody, a secondary enzyme conjugate for quantitation, and a chromogenic or chemiluminescent substrate for the detection of the HCPs (see Eaton 1995 as an example).

An ELISA plate reader measures the intensity of the end product formed. The intensity of the developed product is proportional to the amount of HCPs present in the test article. A standard curve is generated from a cell lysate calibrator of known protein concentration. From a standard curve, the concentration of HCPs in the drug substance is determined. The acceptable amount of HCP in the finished product is based on process capability/performance.

1.9.2.27 Appearance and Color

Each final container is inspected for acceptable appearance and/or color. Depending on the drug product, the appearance of the container (vial, syringe, or sprayer) and content (lyophilized cake or solution) is assessed. This process may be performed by automated visualization or manual observation.

Lyophilized LVV products may also be dissolved using the product-specific diluent, and the color and appearance assessed. Since these products are generally not particulate in nature, they are generally clear, and color may vary from colorless to colored.

1.9.2.28 Endotoxin

This test assures that bacterial endotoxin is not present using the limulus amebocyte lysate (LAL) assay under conditions that are compendial or accepted by regulatory authorities (European Pharmacopoeia (Ph. Eur.), EP 2.6.14; United States Pharmacopeia and The National Formulary, USP <85>).

1.9.2.29 General Safety Testing

General Safety testing is an in vivo test performed to assure that the drug product has no abnormal toxicity when injected into a suitable animal model (European Pharmacopoeia (Ph. Eur.), EP 2.6.9; United States Pharmacopeia and The National Formulary, USP <88>; US Code of Federal Regulations 21 CFR § 610.11). The test article is the final finished container.

Two guinea pigs of a specified weight (e.g., less than 400 g each) and/or two mice of a specified weight (weighing less than 22 g each) are inoculated intraperitoneally with the test article (e.g., 1 dose in a specified volume). Negative controls are injected intraperitoneally with an appropriate placebo (e.g., Hank's balanced salt solution), and all animals are observed daily for 7 days. The test article passes if no overt signs of ill health are observed, no body weight loss is observed at the end of the 7-day observation period, and no deaths occur during the 7-day observation period.

1.9.2.30 Osmolality

If the purified vaccine formulation contains excipients of high ionic strength, the drug product is tested for osmolality (European Pharmacopoeia (Ph. Eur.), EP 2.2.35; United States Pharmacopeia and The National Formulary, USP <785>). Generally, the osmolality of drug product is determined by the freezing point depression of the test article. The osmometer is calibrated with certified standards and verified by measuring the osmolality of a reference vaccine solution. The osmolality test is valid if the reference solution is within the specified range. The osmolality should be within the range demonstrated to be clinically effective in the product license.

1.9.2.31 pH

Appropriately-buffered drug products are essential to maintaining the viability of live attenuated vaccine products. While the pH of liquid product can be measured directly, lyophilized LVV products are reconstituted using the product-specific diluent, and the pH is assessed. The pH meter is calibrated with certified standards and verified by measuring the pH of a reference vaccine solution. The pH should be within the range demonstrated to be clinically effective in the product license. The assay methods are compendial or accepted by regulatory authorities (USP-NF, EP) (European Pharmacopoeia (Ph. Eur.), EP 2.2.3; United States Pharmacopeia and The National Formulary, USP <791>).

1.9.2.32 Water Content (Lyophilized Products)

Water content for lyophilized vaccines is measured to assure that the drug product moisture level is appropriate for maintaining product stability. The moisture content should be within the range demonstrated to confer appropriate stability as indicated in the product license. The assay methods are compendial or accepted by regulatory authorities (European Pharmacopoeia (Ph. Eur.), EP 2.5.12; United States Pharmacopeia and The National Formulary, USP <921>).

1.10 Stability of Starting Materials, Intermediates, Drug Substance, and Drug Product

1.10.1 Stability Testing for Drug Substance

Stability studies are generally conducted on multiple (e.g., three) lots of bulk drug substance manufactured in the licensed facility. The stability of the drug substance is evaluated at different time intervals up to and beyond expiry. The purpose of

Table 1.6 An example of a stability testing schedule for a LVV drug substance with a 36 month expiry

Long-term storage		Months/days ≤ −60 °C							
	Assay	**0**[a]	**3**	**6**	**9**	**12**	**24**	**36**	**39**
	Potency	X	X	X	X	X	X	X	X
	Sterility	X	–	–	–	–	–	X	X

[a] Time 0 data derived from release testing

these studies is to obtain long-term stability data to establish a minimum expiration date (e.g., 24, 36, 48 months) for storage at −60 °C or below (Schofield 2009). This information is also important for considering the drug product filling targets required for drug product manufacturing (filling) described in Sect. 1.8.

An example of a stability testing schedule for a LVV drug substance with a 36 month expiry is shown in Table 1.6. Aliquots of the bulk drug substance are generally stored in an upright position in containers and under conditions that mimic the storage of the bulk product. Stability is assessed at selected time points for potency and sterility. Samples are withdrawn from storage within defined limits from the sample pull date (e.g., ±3 days) with the exception of Time 0, where data are taken from the initial release tests for each monovalent bulk material lot.

1.10.2 Stability Testing for Drug Product

Stability studies are generally conducted on multiple (e.g., three) lots of LVV drug product manufactured in the licensed facility to support licensure and on yearly lots (e.g., three) during the product life cycle. The stability of the drug product is evaluated at different time intervals up to and beyond expiry. The purpose of these studies is to obtain long-term stability data to establish a minimum expiration date (e.g., 6, 12, 24, months) for storage at field conditions (Schofield 2009) to ensure that the claimed dose is administered.

At each point, various attributes such as sterility, potency, pH and appearance of the filled drug product may be measured. In addition, container closure testing is performed to demonstrate container integrity at the end of testing. The time points and temperature of incubation are product specific. The relevant temperatures assessed include the storage temperature of the post-distribution packaged product (e.g., 5 ± 3 °C), and if a different temperature is used pre-distribution (e.g., –15 ± 3 °C), the product is also stored under these conditions. These temperatures mimic the expected field storage condition.

In addition to the licensed storage temperatures, an elevated temperature for determining accelerated stability (e.g., 15 ± 3 °C) is performed. This stability testing evaluates potential excursions in storage, as well as providing information on potency loss that occurs in a shorter period of time (time to stability result; United States Pharmacopeia and The National Formulary, USP <1235>). Finally, samples

Table 1.7 An example of a stability testing schedule for a LVV drug product with 5 ± 3 °C storage and a 24-month expiry

Field storage stability		Months 5 ± 3 °C							
	Assay	**0**[a]	**3**	**6**	**9**	**12**	**18**	**24**	**30**
	Potency	X	X	X	X	X	X	X	X
	Sterility	X	–	–	–	–	–	X	X
	pH and appearance	X	X	X	X	X	X	X	X
	Container closure	–	–	–	–	–	–	X	X
Accelerated stability		Days 15 ± 3 °C							
	Assay	**0**	**1**	**3**	**7**	**14**	**21**	**28**	**35**
	Potency	X	X	X	X	X	X	X	X
Long-term frozen (control)		Months/days ≤ −60 °C							
	Assay	**0**	**3/1**	**6/3**	**9/7**	**12/14**	**18/21**	**24/28**	**30/35**
	Potency	X	X	X	X	X	X	X	X

[a] Time 0 data derived from release testing

are stored frozen (e.g., at −60 °C or less) and function as controls for a minimal (or no) potency loss across the stability testing schedule.

An example of a stability testing schedule for a LVV drug product with a 24-month expiry is shown in Table 1.7. Packaged drug products are stored under conditions that mimic the storage of the product, and stability is assessed at selected time points for potency and sterility.

1.11 Conclusion

Live attenuated viral vaccines have a long history of success spanning over 200 years of use to prevent disease. Their potential to eradicate disease, as was accomplished with smallpox, makes them one of the most significant public health measures available today with the approaching elimination of polio and measles on the horizon. Indeed, their potential utility continues to be appreciated for existing diseases that have no available (commercial) vaccine (e.g., dengue fever, emerging pandemic influenza).

LVV products are complex biologics that require the maintenance of vaccine virus infectivity in order to induce a durable (potentially lifelong) protection from disease. Because of the nature of LVV manufacturing (using viable cell substrates and biological materials such as trypsin and/or bovine serum), extensive safety testing of the raw materials, starting materials, product intermediate, drug

substance, and drug product are critical to ensuring the absence of extraneous agents. However, despite the manufacturing complexity, LVV products have an unparalleled history of safety and effectiveness in the field.

References

Afzal MA, Marsden SA, Hull RM, Pipkin PA, Bentley ML, Minor PD (1999) Evaluation of the neurovirulence test for mumps vaccines. Biologicals 27(1):43–49

Albrecht T, Fons M, Boldogh I, Boldogh I, Rabson AS (1996) Effects on cells. In: Baron S (ed) Medical microbiology, 4th edn. University of Texas Medical Branch at Galveston, Galveston (TX) (Chap 44)

Belser JA, Katz JM, Tumpey TM (2011) The ferret as a model organism to study influenza A virus infection. Dis Model Mech 4:575–579

Boni J, Stadler J, Reigei F, Shupbach J (1996) Detection of reverse transcriptase activity in live attenuated virus vaccines. Clin Diagn Virol 5:43–53

Brandau DT, Jones LS, Wiethoff CM, Rexroad J, Middaugh CR (2003) Thermal stability of vaccines. J Pharm Sci 92(2):218–231

Carter KC (1985) Koch's postulates in relation to the work of Jacob Henle and Edwin Klebs. Med Hist 29(4):353–374

Centers for Disease Control and Prevention (CDC) (1999) Withdrawal of rotavirus vaccine recommendation. MMWR Morb Mortal Wkly Rep 48(43):1007

Chen TR (1977) In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. Exp Cell Res 104(2):255–262

Chen Z, Aspelund A, Kemble G, Jin H (2006) Genetic mapping of the cold-adapted phenotype of B/Ann Arbor/1/66, the master donor virus for live attenuated influenza vaccines (FluMist). Virology 345(2):416–423

Daelemans D, Pauwels R, De Clercq E, Pannecouque C (2011) A time-of-drug addition approach to target identification of antiviral compounds. Nat Protoc 6(6):925–933

Dubin G, Toussaint JF, Cassart JP, Howe B, Boyce D, Friedland L, Abu-Elyazeed R, Poncelet S, Han HH, Debrus S (2013) Investigation of a regulatory agency enquiry into potential porcine circovirus type 1 contamination of the human rotavirus vaccine, Rotarix™: approach and outcome. Hum Vaccin Immunother 9(11):2398–2408

Dulbecco R, Vogt M (1954) Plaque formation and isolation of pure lines with poliomyelitis viruses. J Exp Med 99(2):167–182

Eaton LC (1995) Host cell contaminant protein assay development for recombinant biopharmaceuticals. J Chromatogr A 705:105–114

European Medicines Agency (2013) European medicines agency confirms positive benefit-risk balance of Rotarix. http://www.ema.europa.eu/docs/en_GB/document_library/Press_release/2010/07/WC500094972.pdf. Accessed 22 July 2010

European Pharmacopoeia (Ph. Eur.), EP monograph 01/2013:0537 (2013) Yellow fever vaccine (live), 8th edn

European Pharmacopoeia (Ph. Eur.), EP monograph 01/2012:0215 (2013) Poliomyelitis vaccine (oral), 8th edn

European Pharmacopoeia (Ph. Eur.), EP 2.2.3 (2013) Potentiometric determination of pH, 8th edn

European Pharmacopoeia (Ph. Eur.), EP 2.2.33 (2013) Total protein, 8th edn

European Pharmacopoeia (Ph. Eur.), EP 2.2.35 (2013) Osmolality, 8th edn

European Pharmacopoeia (Ph. Eur.), EP 2.5.12 (2013) Water: semi-micro determination, 8th edn

European Pharmacopoeia (Ph. Eur.), EP 2.6.1 (2013) Sterility, 8th edn

European Pharmacopoeia (Ph. Eur.), EP 2.6.2 (2013) Mycobacteria, 8th edn

European Pharmacopoeia (Ph. Eur.), EP 2.6.7 (2013) Mycoplasmas, 8th edn

European Pharmacopoeia (Ph. Eur.), EP 2.6.9 (2013) Abnormal toxicity, 8th edn
European Pharmacopoeia (Ph. Eur.), EP 2.6.14 (2013) Bacterial endotoxins, 8th edn
European Pharmacopoeia (Ph. Eur.), EP 2.6.16 (2013) Tests for extraneous agents in viral vaccines for human use, 8th edn
European Pharmacopoeia (Ph. Eur.), EP 2.6.18 (2013) Test for neurovirulence of live virus vaccines, 8th edn
European Pharmacopoeia (Ph. Eur.), EP 2.6.24 (2013) Avian viral vaccines: tests for extraneous agents in seed lots, 8th edn
European Pharmacopoeia (Ph. Eur.), EP 2.7.1 (2013) Immunochemical methods, 8th edn
European Pharmacopoeia (Ph. Eur.), EP 2.7.2 (2013) Microbiological assay of antibiotics, 8th edn
European Pharmacopoeia (Ph. Eur.), EP 5.2.2 (2013) Chicken flock free from specified pathogens for the production and quality control of chickens, 8th edn
European Pharmacopoeia (Ph. Eur.), EP 5.2.3 (2013) Cell substrates for the production of vaccines for human use, 8th edn
European Pharmacopoeia (Ph. Eur.), EP 5.2.8 (2013) Minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products, 8th edn
FDA CBER/FDA Points to consider in characterization of cell lines used to produce biologicals (1993)
FDA Guidance for Industry: characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines for infectious disease indications (2006)
FDA Guidance for Industry: characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines for infectious disease indications (2010)
Frierson JG (2010) The yellow fever vaccine: a history. Yale J Biol Med 83(2):77–85
Greenberg HB, Arvin AM (2011) Live attenuated vaccines: influenza, rotavirus and varicella zoster virus. In: Dormitzer PR, Mandl CW, Rappuoli R (eds) Replicating vaccines, a new generation, Birkhäuser in advances in infectious diseases. Springer, Basel, pp 15–46
Hahon N, Booth JA, Eckert HL (1973) Quantitative assessment of hemadsorption by myxoviruses: virus hemadsorption assay. Microbiology 25(4):595–600
Henderson DA, Fenner F (1994) Smallpox and vaccinia. In: Plotkin ST, Mortimer EA Jr (eds) Vaccines, 2nd edn. WB Saunders, Philadelphia, p 1
Hoffmann E, Mahmood K, Chen Z, Yang CF, Spaete J, Greenberg HB, Herlocher ML, Jin H, Kemble G (2005) Multiple gene segments control the temperature sensitivity and attenuation phenotypes of ca B/Ann Arbor/1/66. J Virol 79(17):11014–11021
Hong WS, Yun CR (2001) The medical history of China. Iljong Publisher, Seoul, pp 413–414
Hotchin JE, Cohen SM, Ruska H, Ruska C (1958) Electron microscopical aspects of hemadsorption in tissue cultures infected with influenza virus. Virology 6(3):689–701
ICH, Q5A(R1): viral safety evaluation of biotechnology products derived from cell lines of human or animal origin (1997) http://www.ich.org/LOB/media/MEDIA425.pdf
ICH, Q5D: derivation and characterisation of cell substrates used for production of biotechnological/biological products (1997) http://www.ich.org/LOB/media/MEDIA429.pdf
Jenner E (1798) An inquiry into the causes and effects of the variolae vaccinae, a disease discovered in some of the western countries of England, particularly gloucestershire, and Known by the name of “The cow pox”. R Lier & Co, Milan (1923, p 84)
Jin H, Lu B, Zhou H, Ma C, Zhao J, Yang CF, Kemble G, Greenberg H (2003) Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (FluMist) derived from cold-adapted A/Ann Arbor/6/60. Virology 306(1):18–24
Jin H, Zhou H, Lu B, Kemble G (2004) Imparting temperature sensitivity and attenuation in ferrets to A/Puerto Rico/8/34 influenza virus by transferring the genetic signature for temperature sensitivity from cold-adapted A/Ann Arbor/6/60. J Virol 78(2):995–998
Khan AS, Ma W, Ma Y, Kumar A, Williams DK, Muller J, Ma H, Galvin TA (2009) Proposed algorithm to investigate latent and occult viruses in vaccine cell substrates by chemical induction. Biologicals 37(3):196–201

Killeen KP, DiRita VJ (2001) Live attenuated bacterial vaccines. In: Ellis R (ed) New vaccine technologies. Landes Biosciences, Georgetown, TX, pp 151–170

Kuehn BM (2010) FDA: benefits of rotavirus vaccination outweigh potential contamination risk. JAMA 304:30–31

LaBarre DD, Lowy RJ (2001) Improvements in methods for calculating virus titer estimates from TCID50 and plaque assays. J Virol Methods 96(2):107–126

Lander MR, Chattopadhyay S (1984) A Mus dunni cell line that lacks sequences closely related to endogenous murine leukemia viruses and can be infected by ectropic, amphotropic, xenotropic, and mink cell focus-forming viruses. J Virology 52(2):695–698

Levenbook IS, Pelleu LJ, Elisberg BL (1987) The monkey safety test for neurovirulence of yellow fever vaccines: the utility of quantitative clinical evaluation and histological examination. J Biol Stand 15:305–313

Maudru T, Peden KW (1998) Analysis of a coded panel of licensed vaccines by polymerase chain reaction-based reverse transcriptase assays: a collaborative study see comments. J Clin Virol 11 (1):19–28

Monath TP (1996) Yellow fever vaccines: the success of empiricism, pitfalls of application, and transition to molecular vaccinology. In: Plotkin SA, Fantini B (eds) Vaccinia, vaccination, and vaccinology. Elsevier, Paris, pp 157–182

Murphy TV, Gargiullo PM, Massoudi MS, Nelson DB, Jumaan AO, Okoro CA, Zanardi LR, Setia S, Fair E, LeBaron CW, Wharton M, Livengood JR (2001) Rotavirus intussusception investigation team. Intussusception among infants given an oral rotavirus vaccine. N Engl J Med 344(8):564–572 (Erratum in: N Engl J Med 2001 May 17; 344(20):1564. Livingood JR [corrected to Livengood JR])

Nalca A, Zumbrun EE (2010) ACAM2000: the new smallpox vaccine for United States strategic national stockpile. Drug Des Devel Ther 25(4):71–79

Nissom PM (2007) Specific detection of residual CHO host cell DNA by real-time PCR. Biologicals 35:211–215

Norrby E (2007) Yellow fever and Max Theiler: the only nobel prize for a virus vaccine. J Exp Med 204(12):2779–2784

Onions D, Egan W, Jarrett R, Novicki D, Gregersen JP (2010) Validation of the safety of MDCK cells as a substrate for the production of a cell-derived influenza vaccine. Biologicals 38 (5):544–551

Parks CL, Lerch RA, Walpita P, Wang HP, Sidhu MS, Udem SA (2001) Comparison of predicted amino acid sequences of measles virus strains in the Edmonston vaccine lineage. J Virol 75 (2):910–920

Pasteur M (1881a) An address on vaccination in relation to chicken cholera and splenic fever. Br Med J 2(1076):283–284

Pasteur ML (1881b) On chicken cholera: study of the conditions of non-recidivation and of some other characteristics of this disease. Science 2(33):55–57

Pearce JMS (2002) Louis Pasteur and rabies: a brief note. J Neurol Neurosurg Psychiatry 73(1):82

Plotkin S (2003) Vaccines, vaccination, and vaccinology. J Infect Dis 187:1347–1359

Plotkin S (2008) Correlates of vaccine-induced immunity. Clin Infect Dis 47:401–409

Plotkin SL, Plotkin SA (2004) A short history of vaccination. In: Plotkin SA, Orenstein WA (eds) Vaccines, 4th edn. W.B. Saunders, Philadelphia, pp 1–10

Poon LL, Mak PW, Li OT, Chan KH, Cheung CL, Ma ES, Yen HL, Vijaykrishna D, Guan Y, Peiris JS (2010) Rapid detection of reassortment of pandemic H1N1/2009 influenza virus. Clin Chem 56(8):1340–1344

Ranheim T, Mathis PK, Joelsson DB, Smith ME, Campbell KM, Lucas G, Barmat S, Melissen E, Benz R, Lewis JA, Chen J, Schofield T, Sitrin RD, Hennessey JP Jr (2005) Development and application of a quantitative RT-PCR potency assay for a pentavalent rotavirus vaccine (RotaTeq). J Virol Methods 131(2):193–201

Riedel S (2005) Edward Jenner and the history of smallpox and vaccination. Proc (Bayl Univ Med Cent) 18(1):21–25

Roland KL, Tinge SA, Killeen KP, Kochi SK (2005) Recent advances in the development of live, attenuated bacterial vectors. Curr Opin Mol Ther 7(1):62–72
Rubin SA (2011) Toward replacement of the monkey neurovirulence test in vaccine safety testing. Procedia Vaccinol 5:261–265
Schofield TL (2009) Maintenance of vaccine stability through annual stability and comparability studies. Biologicals 37(6):397–402 (discussion 421-3)
Shahabuddin M, Sears JF, Khan AS (2001) No evidence of infectious retroviruses in measles virus vaccines produced in chicken embryo cell cultures. J Clin Microbiol 39(2):675–684
Sheng-Fowler L, Lewis AM Jr, Peden K (2009) Quantitative determination of the infectivity of the proviral DNA of a retrovirus in vitro: evaluation of methods for DNA inactivation. Biologicals 37:259–269
Siddiqi SH, Libonati JP, Middlebrook G (1981) Evaluation of a rapid radiometric method for drug susceptibility testing of *mycobacterium tuberculosis*. J Clin Microbiol 13:908–912
Siegrist CA (2008) Vaccine immunology. In: Plotkin SA, Orenstein WA, Offit PA Vaccines. 5th edn. Saunders, China, pp 17–36
Theiler M, Smith HH (1937) The use of yellow fever modified by in vitro cultivation for human immunization. J Exp Med 65:787–800
Théodoridès J (1989) Pasteur and rabies: the British connection. J R Soc Med 82(8):488–490
Umino Y, Saito S, Fukuda A, Hishiyama M, Sugiura A (1990) Improvement in potency assay of measles-mumps-rubella trivalent vaccine: interference between components and measures for its elimination. J Virol Methods 27(2):159–168
United States Pharmacopeia and The National Formulary, USP <61> (2014) Microbiological examination of nonsterile products: microbial enumeration tests, 37th edn
United States Pharmacopeia and The National Formulary, USP <62> (2014) Microbiological examination of nonsterile products: tests for specified microorganisms, 37th edn
United States Pharmacopeia and The National Formulary, USP <63> (2014) Mycoplasma tests, 37th edn
United States Pharmacopeia and The National Formulary, USP <71> (2014) Sterility tests, 37th edn
United States Pharmacopeia and The National Formulary, USP <81> (2014) Antibiotics—microbial assays, 37th edn
United States Pharmacopeia and The National Formulary, USP <85> (2014) Bacterial endotoxins test, 37th edn
United States Pharmacopeia and The National Formulary, USP <88> (2014) Biological reactivity tests, in vivo, 37th edn
United States Pharmacopeia and The National Formulary, USP <785> (2014) Osmolality and osmolarity, 37th edn
United States Pharmacopeia and The National Formulary, USP <791> (2014) pH, 37th edn
United States Pharmacopeia and The National Formulary, USP <921> (2014) Water determination, 37th edn
United States Pharmacopeia and The National Formulary, USP <1057> (2014) Biotechnology-derived articles—total protein assay, 37th edn
United States Pharmacopeia and The National Formulary, USP <1235> (2014) Vaccines for human use—general considerations, 37th edn
United States Pharmacopeia and The National Formulary, USP <1237> (2014) Virology test methods, 37th edn
US Code of Federal Regulations 21 CFR § 610.10 Potency
US Code of Federal Regulations 21 CFR § 610.11 General safety
US Code of Federal Regulations 21 CFR § 610.12 Sterility
US Code of Federal Regulations 21 CFR § 610.14 Identity
US Code of Federal Regulations 21 CFR § 610.18 Cultures
US Code of Federal Regulations 21 CFR § 610.30 Test for mycoplasma
Valbuena G, Walker DH (2013) Approaches to vaccines against *orientia tsutsugamushi*. Front Cell Infect Microbiol 2:170

van Elden LJ, Nijhuis M, Schipper P, Schuurman R, van Loon AM (2001) Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. J Clin Microbiol 39(1):196–200
Vesikari T (2012) Rotavirus vaccination: a concise review. Clin Microbiol Infect 18(Suppl 5):57–63
Victoria JG, Wang C, Jones MS, Jaing C, McLoughlin K, Gardner S, Delwart EL (2010) Viral nucleic acids in live-attenuated vaccines: detection of minority variants and an adventitious virus. J Virol 84:6033–6040
Weissmahr RN, Schüpbach J, Böni J (1997) Reverse transcriptase activity in chicken embryo fibroblast culture supernatants is associated with particles containing endogenous avian retrovirus EAV-0 RNA. J Virol 71(4):3005–3012
World Health Organization (1956) Yellow fever vaccination monograph series no 30 (part 2)
World Health Organization (1979) Wkly Epidemiol Rec 54:329
World Health Organization (1980) Global commission for certification of smallpox eradication. The global eradication of smallpox: final report of the global commission for the certification of smallpox eradication. World Health Organization, Geneva
World Health Organization (1998) Wkly Epidemiol Rec 73:105–108
World Health Organization (2007) Expert committee on biological standardization, 56th report, WHO technical report series, no 941
World Health Organization (2010) Statement of the global advisory committee on vaccine safety on rotarix. http://www.who.int/vaccine_safety/committee/topics/rotavirus/rotarix_statement_march_2010/en/
Yang DP, Goldberg KM, Ma XD, Magargle W, Rappaport R (1998) Development of a fluorescent focus identification assay using serotype-specific monoclonal antibodies for detection and quantitation of rotaviruses in a tetravalent rotavirus vaccine. Clin Diagn Lab Immunol 5 (6):780–783
Yeolekar LR, Dhere RM (2012) Development and validation of an egg-based potency assay for a trivalent live attenuated influenza vaccine. Biologicals 40(2):146–150

Chapter 2
Inactivated Viral Vaccines

Barbara Sanders, Martin Koldijk and Hanneke Schuitemaker

2.1 Introduction to Vaccines Based on Inactivated Viruses

The first report of "virus" inactivation for vaccine purposes was described in 1886 when Daniel Elmer Salmon and Theobald Smith immunized pigeons with what they thought was a heat-killed hog cholera "virus" (Salmon and Smith 1886). Although in reality it was a cholera-like bacterium, it seeded the scientific community with evidence that immunization with inactivated pathogens can provide protection against infectious disease. Research continued for at least 15 years when at the beginning of the twentieth century the first killed (bacterial) vaccines for humans were developed for typhoid fever, cholera, and plague (Wright and Semple 1897; Haffkine 1899). The foundations of immunization with inactivated virus preparations were also laid at the end of the nineteenth century with Pasteur's partially inactivated rabies virus (Pasteur et al. 1885), which was cultured in rabbit spinal cords. However, inactivated viral vaccine development was only truly launched with the discovery of cell culture procedures that supported the replication of viral pathogens in vitro, outside the host organism, thus allowing the large scale production of viruses as a source for whole inactivated vaccines. This breakthrough was attributed to Enders, Weller, and Robbins who received the Nobel Prize in 1954 for their discovery on how to cultivate poliovirus in fibroblasts in vitro (Enders et al. 1949; Weller et al. 1949).

In general, all inactivated viral vaccines follow a similar production course in which the pathogen is first cultivated on a substrate to produce large quantities of antigen. Historically, vaccine manufacturers have been using primary cells, tissues,

B. Sanders · H. Schuitemaker (✉)
Infectious Diseases and Vaccines Therapeutic Area, Janssen R&D, Crucell,
Archimedesweg 4–6, 2333 CN Leiden, The Netherlands
e-mail: hschuite@its.jnj.com

M. Koldijk
Crucell Vaccine Institute, Janssen Center of Excellence for Immunoprophylaxis,
Archimedesweg 4–6, 2333 CN Leiden, The Netherlands

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6_2

fertilized eggs, and even whole organisms as substrates for virus propagation (Hess et al. 2012; Barrett et al. 2009). Today, vaccine manufacturers are increasingly shifting toward virus growth on continuous cell lines. This brings certain advantages such as reduced production costs, increased vaccine safety, and relatively straightforward upscaling (Barrett et al. 2009). Once the virus has been propagated, it is often purified and concentrated prior to inactivation. Inactivation can be performed using chemical or physical methods or a combination of the two. A wide range of well-established and novel inactivation agents or methods have been described to successfully inactivate viruses for vaccine purposes. Examples are ascorbic acid (Madhusudana et al. 2004), ethylenimine derivatives (Larghi and Nebel 1980), psorlens (Maves et al. 2011), hydrogen peroxide (Amanna et al. 2012), gamma irradiation (Martin et al. 2010a; Alsharifi and Mullbacher 2010), UV treatment (Budowsky et al. 1981), heat (Nims and Plavsic 2012), and many more (Stauffer et al. 2006). Nonetheless, only formaldehyde and β-Propiolactone (BPL) are widely used for inactivation of licensed human viral vaccines for decades.

Historical events have shaped the way in which inactivated vaccines are currently developed and characterized today. The Cutter incident in 1955 was one of the worst pharmaceutical disasters in the US. history. Here, 380,000 doses of inactivated poliovirus vaccine (IPV), produced at Cutter laboratories, were administered to healthy children. However, these vaccines contained replication competent poliovirus due to inadequate purification of the viral harvest during production. The presence of cell debris in the vaccine pools prevented suitable exposure of the viral particles to formaldehyde and therefore complete inactivation (Offit 2005). As a consequence, 40,000 children who received the vaccine contracted abortive poliomyelitis, 51 were permanently paralyzed, and five died (Nathanson and Langmuir 1963). Federal requirements for vaccine manufacturers were immediately revised in reaction to the Cutter incident creating a better system of regulating vaccines. However, the legacy remains and vaccine manufacturers should always exercise utmost caution when inactivating pathogens to ensure complete inactivation.

Two inactivated vaccines have led to the development of enhanced disease, and even deaths, when the vaccinated persons encountered the pathogen. Indeed, clinical trials with a formalin-inactivated respiratory syncytial virus (RSV) in naïve infants had a disastrous outcome. Not only did the vaccine fail to prevent disease, 80 % of vaccine recipients were hospitalized after encountering circulating RSV as compared to hospitalization of only 5 % in the control vaccine group. Furthermore, two vaccine recipients died as a consequence of the vaccine induced enhanced disease (Kapikian et al. 1969; Kim et al. 1969; Castilow et al. 2007). The enhanced disease was later attributed to an unfavorable immune response due to a skewed Th2 response and lack of antibody affinity maturation after vaccination (Delgado et al. 2009; Johnson and Graham 2004). Furthermore, a large portion of vaccine-induced antibodies were directed to nonprotective epitopes as formalin treatment had altered the epitopes which induce functional (neutralizing and fusion inhibiting) antibodies that are assumed to be required for protection (Murphy and Walsh 1988).

A formalin-inactivated measles vaccine was licensed in 1963 and unfortunately resulted in a similar, albeit less severe, outcome. The vaccine did induce neutralizing

antibodies, however, immunity waned rapidly and recipients regained susceptibility to measles. When contracted, a more severe, atypical measles disease developed (Griffin and Pan 2009). As with RSV, the enhanced disease was associated with a lack of cytolytic T-cell response and low avidity antibodies (Polack et al. 1999, 2003), linked to the formaldehyde-induced alteration of the measles F protein (Annunziato et al. 1982). In general, it has been suggested that the carbonyl groups on vaccine antigens introduced by formaldehyde treatment induce profound effects on immunogenicity which may tip the balance between protection and adverse effects, or enhanced disease (Moghaddam et al. 2006). These unfortunate events serve as a warning to all vaccine developers; inactivation of a pathogen does not necessarily translate into a vaccine that by default elicits protective immunity, viral epitopes necessary for induction of protective immunity should be preserved after inactivation.

These past events have shaped the manufacture and regulatory control of inactivated vaccines today resulting in vaccines with extremely high safety profiles which protect millions of people against a range of pathogens. The realization that inadequately inactivated viruses could result in such tragedy instigated the need for the exertion of control over the production process. It is for this reason that today inactivation processes require vigilantly designed and validated inactivation assays to ensure pathogens are inactivated with utmost certainty. Concurrently, the successfully formulated inactivated viral vaccines on the market today have a heightened safety aspect as compared to live attenuated viral vaccines. The fact that the pathogen is completely inactivated directly negates reversion to a virulent phenotype within the vaccine recipient. Moreover, these inactivated vaccine viruses are non-transmissible as their live attenuated counterparts. Indeed, reversion and transmission are the greatest drawbacks of attenuated vaccines and have been observed in the past with the attenuated poliovirus vaccine (Henderson et al. 1964), yellow fever vaccine (Lindsey et al. 2008), and rotavirus vaccine (Patel et al. 2009). The improved safety profile of inactivated vaccines entails that they are also suitable for the rapidly increasing group of immunocompromised individuals (Ljungman 2012).

To achieve such a high degree of safety, the analyses of virus inactivation are crucial for production of an inactivated vaccine. The kinetics of inactivation (KOI) must be completely understood and to ascertain the completeness of inactivation the test for effective inactivation must be validated and well characterized with respect to sensitivity and robustness. The KOI will differ per pathogen and inactivation method, therefore, to ensure the safety of the inactivated vaccine bulk, the inactivation process should be studied extensively, where observation of a reproducible KOI is essential. Quantification of viral infectivity in either the vaccine bulk or in-process intermediates is usually achieved by an in vitro cell culture-based assay, however this can also be done in vivo. Generally, either the cell line used for virus propagation or an alternative cell line, demonstrated to be equally susceptible, is inoculated with the (inactivated) virus sample to amplify any potential infectious unit present. Presence or absence of virus in in vitro cultures can be detected by various methods; for lytic viruses this is enabled by monitoring of cytopathic effect (CPE), for nonlytic viruses methods based on genome amplification (PCR) or

antigen detection (immune-fluorescence or ELISA) can be used. In addition, a second step involving inoculation of amplified material in an appropriate in vivo model followed by monitoring for the onset of disease symptoms, can be performed.

Since testing for effective inactivation is dependent on assay sensitivity, sample volume, and the absence of interference by inactivated particles, the assay used to confirm completeness of inactivation (COI) should be designed to be easily scaled up, with high sensitivity and rigorous controls for assay sensitivity and matrix effects. Specifically, positive controls including samples spiked with a known concentration of virus to confirm susceptibility of the cell cultures. Furthermore, negative matrix controls are taken along to ensure no other components in the formulation induce cell death or interfere with assay sensitivity. When making an inference on the COI, two variables play a role. The first one relates to the sensitivity of the COI assay which must be characterized to assign a minimum number of infectious units (i.e., lower limit of detection) that can be detected using the assay. The second one relates to the sampling size; the larger the volume of the test sample, the higher the chance of detecting a potential infectious unit in the entire batch. The combination of these two variables allows the manufacturer to either specify a maximally tolerable level of outgoing infectivity (Cornfield et al. 1956) or adhere to a predefined criterion. The World Health Organization (WHO) and European Pharmacopoeia (Ph. Eur.) have stipulated guidelines for testing for effective inactivation with a minimum sample, expressed in volume or number of doses, which must be tested. Furthermore, cell types, duration of incubation, and dilution of vaccine sample prior to inoculation are parameters that can influence the sensitivity of the COI and thus must be thoroughly optimized. The testing for COI for the different vaccines will be discussed per vaccine in more detail later in this chapter.

Once inactivated, the viral bulk is typically further purified to remove contaminants, this can be achieved by utilizing various techniques, examples are: ultrafiltration, size-exclusion chromatography (SEC), and sucrose gradient centrifugation. Furthermore, tests are required to assess the purity of the vaccine product, such as testing for the absence of contaminants arising from the production process such as host cell protein and DNA. Additionally, to ensure no modification of epitopes occurs during inactivation, as was observed with RSV and measles in the past, the immunogenic potency of the inactivated virus particle must be measured. This can be achieved by measuring the immune response before and after immunization in vivo, for example the Rat potency assay which is used to measure poliovirus neutralizing antibodies after immunization of rats with an inactivated poliovirus vaccine (van Steenis et al. 1981). In vivo immunogenicity testing can also been correlated to in vitro cell-based potency assays; such as the D-antigen ELISA which quantifies the antigenic content of inactivated poliovirus particles and is consequently used for dosing of the vaccine (Beale 1961). Different in vitro quality control systems to monitor antigenic integrity of an inactivated vaccine have been developed for rabies (Rooijakkers et al. 1996a, b), influenza (Di Trani et al. 2003), poliovirus (Morgeaux et al. 2005), and hepatitis A virus (Poirier et al. 2010). Naturally, the

development of such assays requires knowledge of the neutralizing epitopes necessary for an adequate immune response, and consequently, protection.

Not only do inactivated vaccines possess a higher safety profile as compared to live vaccines, they are also generally less reactogenic, relatively straightforward, and technically feasible to produce with fewer regulatory hurdles for licensure (Zepp 2011). However, inactivated vaccines are typically associated with a lower immunogenicity which can imply the necessity of multiple doses or adjuvant addition which consequently raises the costs of goods and vaccine pricing. Therefore, choosing an inactivated vaccine approach is in general a trade-off with on one hand increased safety (if inactivation is of course complete) and a fast pathway to regulatory approval, but on the other hand the risk of reduced antigenicity of the immunogen which often requires adjuvant addition and/or multiple doses which not only raises production costs but also the complexity of formulation and administration.

Today, there are six licensed viral vaccines that are inactivated with either formaldehyde or BPL. Formaldehyde is used for the inactivation of Poliovirus (PV), Hepatitis A Virus (HAV), Japanese Encephalitis Virus (JEV), and Tick Borne Encephalitis Virus (TBEV) to generate vaccines. BPL is used for the inactivation of Rabies and Influenza virus vaccines, however, there are also licensed vaccines against these infections that use formaldehyde as inactivating agent. This chapter will focus on the background, inactivation procedures, and analyses of inactivation of the six currently licensed inactivated viral vaccines categorized per inactivation method. Furthermore, attention will be paid to new inactivated viral vaccines in development.

2.2 Inactivated Vaccines Based on Formaldehyde Inactivation

Formaldehyde is the most widely used inactivating agent for vaccine purposes and many pathogens have been subjected to the irreversible modifications formaldehyde inflicts by cross-linking of various amino acids. Here, we describe the history, kinetics, and mechanism of formaldehyde inactivation, and subsequently the vaccines which currently use this inactivation agent.

For the sake of clarity and ease of referencing, nomenclature referring to either formaldehyde or formalin will reflect the terminology used by the original authors. For reference, 37 % w/v formaldehyde (13.3 M) equals 100 % formalin and a 1/4,000 dilution of formalin is thus identical to 0.009 % formaldehyde (i.e., 3.3 mM or 100 μg/ml formaldehyde).

Formaldehyde, with chemical formula CH_2O, is the simplest member of the aldehydes, a group of organic compounds containing a carbon double bonded to hydrogen and a varying side chain. Formaldehyde exerts its effect by a great diversity of modifications (methylol groups, Schiff bases, and methylene bridges)

and the precise mechanisms are subject of investigation in several recent studies (Metz et al. 2004, 2006; Thaysen-Andersen et al. 2007). These modifications culminate in inactivation, stabilization, or immobilization of proteins with consequent loss of viral infectivity.

The first successful use of formaldehyde treatment was in the field of toxin inactivation. Here, Glenny and Hopkins accidentally discovered (Brown 1995) that residual traces of formalin—used for cleaning—rendered batches of diphtheria toxin non-toxic in guinea pigs (Glenny and Hopkins 1923). At the same time Ramon demonstrated that diphtheria toxoid, prepared by incubation with formalin at 37 °C for several weeks, retained its antigenic qualities (Rappuoli 1994; Wassilak et al. 2004). A similarly developed tetanus toxoid by Ramon and Zoeller was used to vaccinate humans against tetanus in 1926 (Wassilak et al. 2004). The form of the toxin which results from denaturing with formaldehyde is called an anatoxin, "toxoids", or "formalin-toxoids", and is used in vaccinations worldwide. Following the discovery of diphtheria and tetanus toxoids, formaldehyde was applied in the 1930s for the preparation of whole inactivated viral vaccines by inactivation of Russian Autumnal Encephalitis (Halstead and Tsai 2004), (a synonym for JEV), and TBEV (Smorodintsev and Ilyenko 1960).

The development of a formalin-IPV in the 1950s by Salk followed the previous attempts undertaken in the 1930s which had resulted in paralysis and several deaths due to incomplete inactivation of active virus (Brodie and Park 1936). At the time, it was largely believed that long lasting immunity required the administration of a live vaccine for successful immunization and protection, as was observed with the live attenuated small pox and yellow fever vaccines. However, Salk's prior experience with formalin-inactivated influenza (Salk and Francis 1946) prompted him to believe that inactivated vaccines were possible and stimulated his research on PV inactivation using formalin. Aware of the disastrous events that came to pass with the first generation of inactivated PV vaccines, Salk vigilantly investigated formalin inactivation to guarantee safety of the vaccine preparations and established the principle of inactivation which started with the description of PV inactivation in 1953. At low temperatures the activity of formaldehyde is relatively low and might, thereby, be more selective in destroying infectivity without impairing too greatly the antigenic activity of the virus. Salk therefore inactivated PV using a 1/250 dilution of 37 % formaldehyde (corresponding to a final concentration of 0.4 % formalin) at the temperature of melting ice (Salk 1953). In the meantime, virus cultivation methods improved (Farrell et al. 1955) and higher titers of virus required up to 3 weeks of inactivation (Salk et al. 1954). To be able to inactivate also more potent batches of virus, Jonas Salk modified his inactivation process, now using a higher temperature (35–37 °C) and a lower concentration of formalin (1/4,000–1/8,000). The resulting process demonstrated linear inactivation kinetics and was able to inactivate also more potent batches of PV in a comparable timeframe (Salk et al. 1954; Salk and Gori 1960). Figure 2.1 is a graphical representation of an inactivation kinetics curve and illustrates Salk's determination of the minimum required inactivation time.

A different approach was advocated by Swedish scientist, Sven Gard. In his experience, the thermal inactivation of PV at 37 °C was in the order of magnitude of

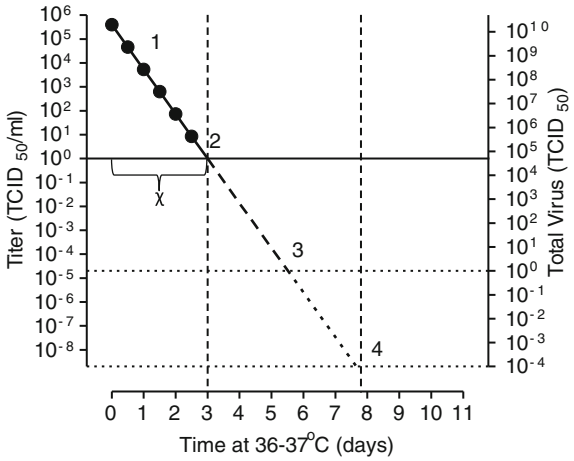


Fig. 2.1 Inactivation kinetics of poliovirus at 36–37 °C according to Salk's first-order hypothesis (Salk et al. 1954). The rate of destruction of infectivity in the bulk is followed by determining, for example, viral titers (Tissue Culture Infectious Dose 50 %, $TCID_{50}$) (*1*). A straight line is drawn through these experimentally attained data points and extrapolated to the point indicating complete absence of infectivity at the intercept of x-axis (*2*). Followed by taking into account the total volume (i.e., 50 L) to be inactivated (*3*) and a safety margin to allow for imperfections in the sensitivity of the tissue culture system used for detection of residual replication competent virus (*4*), the total inactivation time is defined as a total period equal to three times the interval required for interception of the baseline (χ) which would, in this example, correspond to an incubation time of 9 days

1–2 log units in 3 days ($^1/_3$–$^2/_3$) log per day). With the aim of eliminating the thermal component from the PV inactivation, Gard selected 25 °C where the thermal inactivation was only 0.3 log units in 5 days (0.06 log per day), together with 0.006 M formaldehyde (Gard 1957a, b). However, using these conditions a significant deviation from Salk's linear relationship was observed (Gard 1957a, b; Gard and Lycke 1957; Gard et al. 1957; Lycke et al. 1957). To date, these observations are referenced to as evidence that the kinetics of formaldehyde inactivation is not linear or first-order (Bahnemann 1990; Brown 1993). In fact, whereas the kinetics of inactivation did not follow a first-order inactivation when performed at 4 or 25 °C, no deviation from first-order was observed at 37 °C (Salk and Gori 1960). Similarly, the inactivation of HAV—a process based on the principles of Salk—for the VAQTA vaccine showed no evidence of departure from linearity through 7 logs of inactivation (Armstrong et al. 1993).

A key consideration of Gard for selecting a lower inactivation temperature than Salk was the prevention of thermal inactivation of PV. In 1957, Charney et al. (1957), studied both formalin and thermal inactivation at 37 °C. Their results indicated that, contrary to the previously perceived thermal inactivation of 0.3–0.6 log per day (Gard 1957a, b), the thermal inactivation could be limited to 0.2 log per day and the addition of cations further improved thermal stability of PV

(Melnick 1991). Specifically, the addition of 1 M of $MgCl_2$ prevented thermal inactivation without an effect on the rate of formalin inactivation (Ozaki and Melnick 1963).

Having established the conditions for PV inactivation, Salk formulated the principles of inactivation of PV (Salk et al. 1954) which are still used as the basis for the development of inactivated vaccines (for a comprehensive overview see Pittman and Plotkin 2004). Briefly, the rate of destruction of infectivity in the bulk is followed by measuring for example the viral titer over time, which is measured by observing the (lack) of cytopathic effect in cells inoculated with the sample. When taking samples during inactivation, it must be taken into account that the formaldehyde present in the in-process sample must be neutralized prior to titration, for example, by addition of sodium bisulfite. Once viral titers during inactivation are obtained a straight line is drawn through these points and extrapolated to the point that indicates complete absence of infectivity. By taking into account the volume of the bulk that is being inactivated and a safety margin, the inactivation time can be defined as a total period equal to three times the interval required for interception of baseline (Salk et al. 1954). This principle is still used to define the minimum time that is required for complete inactivation (Armstrong et al. 1993; WHO 2002; Plotkin and Vidor 2004). However, studies investigating the effect of prolonged inactivation—treatment with formaldehyde beyond the time that is required for complete destruction of infectivity—showed that after a certain time, antigenicity of the material will decline (Salk and Gori 1960). Specifically, overtreatment of PV is defined as a period equal to more than fivefold the time required to reduce infectivity beyond the point at which it cannot be measured (Salk 1955). In summary, the inactivation time for PV can be defined as at least 3X but not more than 5X.

The use of formaldehyde for the inactivation of PV has one particular phenomenon that was discovered after Dulbecco demonstrated in 1954 that the number of plaques obtained for the three poliomyelitis viruses using monolayer tissue of monkey kidney and monkey testis was proportional to the concentration of virus (Dulbecco and Vogt 1954). Using this technique, Schultz et al. and Böttiger et al., demonstrated in 1957 and 1958, that formalin treatment alone produces a delay in initiating infection of tissue cultures as evidenced by the rate of appearance of plaque formation. Since the delay is progressively greater with extended formalin treatment, it becomes more and more important to prolong the observation period as complete inactivation by formalin is approached (Schultz et al. 1957; Bottiger et al. 1958). Consequently, large scale tissue culture safety tests include extended incubations—up to 35 days (Beardmore et al. 1957) for the detection of replication competent virus. Noteworthy here is that fully active PV could be detected within 14 days whereas false negatives occurred with formalin treated virus if not for the 21- and 28-days subcultures (Beardmore et al. 1957). Consequently, design of methods for demonstrating completeness of inactivation of formalin-inactivated virus should include verification and challenging of incubation times not only with fully active virus but also with formalin treated virus. In conclusion, current guidelines recommend to continue cultures for the detection of residual replication competent virus for as long as technically feasible (WHO 2002).

After completion of the inactivation period, it is common practice to demonstrate that the inactivating agent is active and therefore not rate limiting at the end of inactivation. One way of demonstrating inactivating capacity at the end of the incubation period is by spiking a sample with a known concentration of infectious virus and showing an additional reduction over time in titers of replication competent virus. Alternatively, one can estimate the concentration of residual free formaldehyde. There are a number of methods available for the determination of residual free formaldehyde in inactivated vaccines which are based on Hantzsch, MBTH, Phenylhydrazine, Tryptophan, Chromotropic acid, and Schiff reagents (Frerichs and Chandler 1980; Chandler and Frerichs 1980). Whereas the different methods give comparable results with non-neutralized formaldehyde, only the MBTH, Phenylhydrazine, and Schiff reagent method were accurate with vaccines in which formaldehyde was neutralized with sodium bisulphate (Chandler and Frerichs 1980) and of these three methods, the MBTH method is currently prescribed by the Ph. Eur. (2011a) for sodium bisulfite neutralized samples.

2.3 Examples of Formaldehyde Inactivated Vaccines

2.3.1 Inactivated Picornavirus Vaccines: IPV and HAV

Today, two licensed vaccines that are directed at PV and HAV, both members of the picornaviridae family, are based on formaldehyde inactivated whole viruses. The IPV was first licensed in 1955 while it took another 40 years before a HAV vaccine became available on the market. This delay was primarily due to the inability to propagate HAV to high titers in cell culture. Nonetheless, these two picornavirus vaccines have a comparable production method, where the well-established inactivation and testing of IPV has been used as a benchmark for the HAV vaccine inactivation and testing thereof.

Salk's extrapolation of the linear regression of viral titers to ascertain completeness of inactivation to undetectable levels of infectivity resulted in a minimum inactivation time defined as 3X (Fig. 2.1), which included an incorporated safety margin for complete vaccine safety (Salk et al. 1954), as described earlier. Today, the WHO requires a similar extrapolation of inactivation kinetic curves to ensure sufficient inactivation time, as Salk proposed in the 1950s. The WHO stipulates a thoroughly studied inactivation curve where the inactivation time used for manufacture must exceed the time taken to reduce the virus titer to undetectable levels by at least a factor of 2 (WHO 2002). After inactivation the bulk must also be tested for completeness of inactivation, which must be done with sufficient rigor (Bodian 1958) as administration of incompletely inactivated vaccines is an unacceptable consequence for any manufacturer or regulatory body.

Testing for completeness of inactivation necessitates fixing a maximally tolerable level of outgoing infectivity, but this level need not necessarily ever be realized (Cornfield et al. 1956). The test strategy is based on detection of very low

concentrations of virus; at low concentrations where it is evident that an aliquot representing only a small percentage of the overall bulk may not contain infectious virus (USFDA 1998). The probability p that a sample does not contain virus can be expressed as:

$$p = \left(\frac{V - v}{V}\right)n$$

where n is the absolute number of virus particles distributed in the overall volume (V) of the sample and v is the volume of the aliquot taken for residual virus testing. The above equation can be approximated by the Poisson distribution when a small aliquot of the overall sample is taken $V \gg v$:

$$p = e^{-cv}$$

$$c = (\ln p)/ - v$$

where c is the concentration of infectious virus particles per liter. Together with assumption on viral dispersion in the vaccine bulk, sensitivity of the detection system, and the viral concentration capable of inducing infection in man, one can determine an acceptable volume to test for a satisfactory probability of complete inactivation (Cornfield et al. 1956; Meier 1957). Therefore, the probability of detection of infectious virus at various virus concentrations per liter can be calculated, and upon assigning values for c and p one can calculate the testing volume necessary to measure virus at low levels with high probability of true detection. For example, a report published in 1956 by the US Public Health Service (Cornfield et al. 1956) recommended that 1,500 mL be tested from each of the single strain bulks on the basis of an "acceptable" level of infectivity set at an arbitrary 5 particulates per liter (White 1955). This means that with a bulk containing 5 or more particulates per liter, sampling 1,500 mL of this bulk should result in a probability of ≥99.9 % that the sample will contain ≥1 replication competent virus which should be unequivocally positive in the detection system.

Today, WHO and Ph. Eur. continue to adhere to the statistical sampling described in the 1950s, and consequently the testing for completeness of inactivation of IPV requires two samples that are equivalent to 1,500 adult doses to be tested in vitro on a susceptible cell type. Absence of PV infection in cell tissue cultures inoculated with these doses for at least 3 weeks is required before the monovalent bulk can be released for vaccine formulation (Ph. Eur. 2011d; WHO 2002).

The decades of research and experience with IPV has lead to its role as benchmark for the formalin inactivation of other pathogens in the vaccine field. The lessons learned in the beginning and middle of the last century had paved the way for the formulation of the Hepatitis A picornavirus vaccine. As with IPV, the manufacturability of a Hepatitis A vaccine required the successful propagation of the virus in a suitable substrate. The breakthrough of HAV growth in MRC-5 and WI-38 cell culture was achieved at the end of the 1970s by Maurice Hilleman et al.

(Provost and Hilleman 1979). Not only were these strains adapted to cell culture to allow large scale vaccine production, they had also lost their virulence during cell culture adaptation and were considered attenuated. In fact, their use as live vaccines has also been investigated (Midthun et al. 1991; Provost et al. 1986a). Nonetheless, development of an inactivated vaccine was favored due to the faster route to regulatory approval, resulting in a safe and effective formalin-inactivated vaccine in 1986 (Provost et al. 1986b).

Table 2.1 gives an overview of the currently licensed Hepatitis A vaccines. Manufacturing of these vaccines follows the general flow of events for all inactivated vaccines; virus propagation, harvest and purification, inactivation, further purification, and testing for residual replication competent virus. All manufacturers use the diploid human MRC-5 cell line for the propagation of different Hepatitis A strains of which HM175 and CR326 have been shown to be highly attenuated. Purification and concentration are followed by inactivation. Specifications for inactivation conditions such as formaldehyde concentration, temperature and length of inactivation, are undefined. Instead a manufacturer validated inactivation procedure is used, with elucidation of inactivation kinetics and extrapolation of the curve where 100 % inactivation is achieved and multiplication of this inactivation time by a factor 3 is required, as is the case for IPV (Ph. Eur. 2011b; WHO 1995). The inactivation kinetics of Merck's VAQTA vaccine have been described in the literature and show linear inactivation kinetics for formaldehyde at various formaldehyde concentrations tested. Formaldehyde at a concentration of 100 μg/ml and

Table 2.1 Overview of inactivated Hepatitis A vaccines and their properties

Name	Havrix	Vaqta	Avaxim	Epaxal
Manufacturer	GSK	Merck	Sanofi Pasteur	Crucell Switzerland
Virus strain	HM175	CR326	GBM	RG-SB
Cell substrate	MRC5	MRC5	MRC5	MRC5
Concentration and purification	Sterile filtration, ultrafiltration, gel permeation chromatography	Precipitation in polyethylene glycol, chromatography	Sterile filtration, chromatography, ultrafiltration, diafiltration	Ultrafiltration, ultracentrifugation
Inactivation parameters	250 μg/ml formaldehyde 15 days at 37 °C	100 μg/ml formalin 20 days at 37 °C	Formaldehyde concentration not specified 14 days at 37 °C	0.25 % formalin (w/v) 10 days at 37 °C
Adjuvant	Aluminum hydroxide	Aluminum hydrophosphate	Aluminum hydroxide	Immunopotentiating reconstituted influenza virosomes (IRIV)
Antigen per dose for adult and child doses	1440 ELISA units ≥19 years	50 HAV Antigen units ≥19 years	160 Antigen units >15 years	>24 IU HAV protein ≥2 years
	720 ELISA units 2–18 years	25 HAV Antigen units 2–18 years	80 Antigen units <16 years	
Reference	Andre (1995)	Armstrong et al. (1993)	Vidor et al. (1996)	Gluck et al. (1992)

at a temperature of 37 °C reduced infectious titers to 1 ($TCID_{50}$)/ml within 48 h (Armstrong et al. 1993). The reduction in infectivity is quantified by titration in susceptible cells (i.e., MRC5). During manufacturing of VAQTA, HAV is inactivated during 20 days, which is tenfold the time needed to reach the x-intercept. This process thus exceeds by far the threefold inactivation time specified by regulatory authorities; the authors suggest that prolonged incubation of HAV with formaldehyde does not compromise the immunogenicity of the virus particle, which is not the case for PV, as described earlier.

A manufacturer's validated and thoroughly investigated inactivation procedure is evaluated for approval by the regulatory authorities. As with IPV, testing for effective inactivation relies on reproducible inactivation kinetics and the testing of two samples of 1,500 doses on a suitable substrate, the identical number of doses as used for the testing of inactivated PV (Ph. Eur. 2011b; WHO 1995).

In contrast to IPV, the Hepatitis A vaccine includes the addition of an adjuvant for adequate immunogenicity. Most manufacturers of Hepatitis A vaccines have utilized the well characterized and established aluminum salts as adjuvants that are thought to strengthen humoral immune responses (Gupta 1998). An exception is Epaxal, manufactured by Crucell, in which the inactivated Hepatitis A particles are adsorbed to virosomes of Influenza haemagglutinin, which are argued to stimulate both cellular and humoral immune responses (Bovier 2008; Bungener et al. 2005).

2.3.2 Inactivated Flavivirus Vaccines: JE and TBE

Two formalin-inactivated vaccines are available for protection against infection with the flaviviruses JEV and TBEV. JEV is most prevalent in Asian regions (Gupta 1998) whereas TBE can be encountered in European to Asian regions (Rendi-Wagner 2008). Due to their limited distribution, vaccines against these viruses are not produced in quantities for global immunization but instead at quantities that can supply inhabitants of endemic areas, as well as travelers and expatriates (WHO 2006b, 2011). Manufacturing of these vaccines occurs mainly in the endemic areas by local manufacturers; therefore regulations for safe vaccine production are largely left to the scrutiny of the national regulatory authority. As is the case for HAV vaccines and IPV, global regulatory authorities such as WHO and Ph. Eur. do not specify any inactivation conditions. Instead manufacturers are again expected to demonstrate extensive studies on the inactivation of JE and TBE virus by using, for example, formalin to create safe vaccine batches. The local national authority would then determine whether the vaccine inactivation procedure is sufficient to guarantee a safe, completely inactivated vaccine. The WHO does, however, give examples of methods that have been used for the inactivation of JEV and TBE vaccines; for JEV this is stated as 50–60 days at 4 °C with a formaldehyde concentration of 1 in 2000 (WHO 2007b) although for a Vero-based JEV vaccine inactivation during several months at 4 °C with a higher formalin concentration of 0.08 % was used (Sugawara et al. 2002). For TBE manufacture a formaldehyde

concentration of 0.05 % is proposed with an inactivation time of 5 days at 22 °C (WHO 1999). For WHO approval, testing for completeness of inactivation requires testing of 25 human doses for JEV (WHO 2007b) and 20 doses for TBE (WHO 1999), while the Ph. Eur. requires a minimum of 10 human doses for TBE (Ph. Eur. 2011f). The vaccine doses are to be amplified on a suitable cell substrate followed by intracerebral inoculation of mice with the resulting culture fluid.

2.3.3 Formaldehyde Vaccines Summarized

Methods for formaldehyde inactivation vary greatly between vaccines. Differences lie in formalin concentrations (from 0.08 to 0.009 % w/v), time of inactivation (from days to months), and temperature (usually 4 or 37 °C). In general, the higher the formalin concentration and temperature the faster the inactivation, but this may come at a cost on immunogenicity as thermal degradation and destruction of important epitopes upon higher formalin concentration is a known phenomenon. Therefore, an inactivation time must be sufficient to ascertain complete inactivation but not too long as to destroy immunogenicity, concurrently, a manufacturer should monitor immunogenicity of the inactivated sample during inactivation to ensure no loss of antigenic potential. When faced with the development of an inactivated viral vaccine, an in depth understanding of the particular pathogen's inactivation kinetics curve is inevitable as well as the necessity for a robust test for completeness of inactivation. Only then a reproducible and validated inactivation procedure can be set in place to ensure that a negative result in the test is a true measure for the absence of replication competent virus in a vaccine batch.

2.4 BPL Inactivation of Viruses for Vaccine Purposes

β-Propiolactone (BPL) is the second agent that is widely used for the inactivation of viruses in order to use them as vaccines. BPL is used in the production of Influenza and Rabies vaccines but is also used for vaccines that are currently being developed.

BPL, a colorless liquid with a slightly sweet odor, is part of the four-member ring lactones family. The chemical reactivity of the almost planar, energetically highly strained four-membered ring provides the organic compound with its electrophilic nature and thus the ability to react readily with nucleophiles. While being stable in concentrated liquid form, in aqueous solutions it is unstable due to rapid hydrolysis that allows it to react with hydroxyl, amino, carboxyl, sulfhydryl, and phenolic groups (Hartman and Logrippo 1957). The reactions of BPL with all solution constituents make BPL in essence a self limiting compound. The rapid hydrolysis into nontoxic, noncarcinogenic products will completely eliminate BPL levels from the reaction within 2 h at 37 °C. This gives BPL an advantage over formaldehyde inactivation where residual formalin must be removed. In addition to

its self-limiting nature, excess BPL can be neutralized by the addition of thiosulphate, for example, when sampling virus during inactivation. This method is preferred over a high temperature spike for the neutralization of BPL activity as the latter may have the unwanted side effect of thermal degradation of the virus (Lawrence 2000). All reactions with BPL are rapid and stable. Alkylation or acylation reactions with interacting nucleophiles, which are repeatedly present in large biological macromolecules such as DNA and RNA, are irreversible.

BPL was first produced in 1915 by Johannson (Hartman and Logrippo 1957) who studied the salt of β-iodopropionic acid. However, it was not until 1941 when a novel method of synthesis was introduced by Kung to form BPL from ketene and formalin, that the widespread use of this organic compound began (Hartman and Logrippo 1957). After the discovery of this novel production method, BPL was subject to extensive characterization and research leading to the chemical's fast introduction into multiple industries with varying roles. BPL has been used as a sterilizing agent for tissue grafts and plasma, a monomer for the plastic polymerization industry, an intermediate in the synthesis of propionic compounds, and a virucidal inactivating agent for vaccine purposes (Hartman et al. 1954; Lawrence 1999). The extensive use of BPL in these industries was somewhat reduced when BPL's carcinogenic nature was recognized. Rightfully so, the use of carcinogens in production processes is considered a safety hazard (Hueper 1963). Nonetheless, BPL has remained essential for the production of the abovementioned licensed BPL-inactivated vaccines as levels of BPL are completely hydrolyzed during vaccine production and residual levels verified.

The mechanism of action by which BPL can inactivate viruses is thought to lie primarily in its direct interaction with nucleic acids (Colburn et al. 1965; Mate et al. 1977; Roberts and Warwick 1963). The alkylation and acylation reactions between nucleotides and BPL have been extensively studied. It has been shown that BPL mainly reacts with the Nitrogen-7 atom of guanosine, and to a lesser extent with adenosine at the N_1 position (Hemminki 1981). The BPL-modified guanine is then misread by the polymerase as an adenine, therefore for every alkylated guanosine a GC-AT transition mutation is incorporated (Segal et al. 1981). These multiple mutations in combination with BPL-induced DNA double helix cross-linking (Perrin and Morgeaux 1995) can render the genomes dysfunctional, ultimately making the pathogen replication incompetent, leading to its complete inactivation. As BPL primarily interacts with DNA or RNA, it was assumed that the immunogenic epitopes of the protein would remain intact; entailing that BPL-inactivated viruses would maintain their high immunogenicity, which is not always the case for formaldehyde inactivated viruses. However, amino acids and proteins also display nucleophilic moieties with which BPL can readily interact, and so, as with formaldehyde, the interaction of BPL with viral proteins could induce conformational changes on the viral surface. This could result in the alteration of epitopes necessary for the induction of neutralizing antibodies against the pathogen. Uittenbogaard et al. have elucidated the alkylation or acylation modifications that BPL induces on amino acids. They have shown that BPL reacts with nine different amino acids (Uittenbogaard et al. 2011) which implies that BPL inactivation could result in

alteration of important immunogenic epitopes necessary for an immune response. However, BPL will react more readily with nucleotides in DNA and RNA than with amino acid moieties. BPL mediated alkylation or acylation of viral constituents induces significant modifications of these complex molecules which prevents viral replication, upon which the virus particle is considered to be inactivated (Dijkstra 1975; Logrippo 1960; Roberts and Warwick 1963). Overall, the impact of BPL on immunogenicity varies per pathogen but is lower than the impact of formalin as BPL interacts with protein moieties to a lesser extent.

Viral inactivation is directly correlated with the nature of the virus, the initial concentration of BPL, the temperature, and the composition of the solution during inactivation (Budowsky et al. 1991). For vaccine production, these parameters are generally set at 4 °C for 18–24 h with a BPL concentration of 0.1–0.25 %, however, this can differ per pathogen (Lawrence 2000). Understanding of BPL inactivation kinetics is essential to ensure complete vaccine safety which dictates a reduction of infectivity of initial virus suspension by 15–20 orders of magnitude. This is based on various factors such as minimum probabilities of viral presence in a whole batch and doses necessary for immunization (Budowsky and Zalesskaya 1991). The magnitude of 15–20 orders of inactivation cannot be determined experimentally but requires extrapolation of kinetics data obtained from the experimentally detectable parts of the inactivation kinetics curves (Budowsky and Zalesskaya 1991). Viral inactivation curves after exposure to BPL are not linear and display a phenomenon called "tailing": a reduction in inactivation rate during BPL treatment. This is due to the decrease in BPL concentration over time that is caused by the reaction of the agent with virus, water, or medium components, which decreases the rate of inactivation over time. The kinetics of viral inactivation can be described rather accurately when the varying BPL consumption rates over the reaction are taken into account using the following formula:

$$\ln\frac{S_0}{S_t} = \frac{k}{k_1}A_0[1-\exp(-k_1 t)] \text{ (Budowsky et al. 1991; Budowsky and Zalesskaya 1991)}$$

where S_0 and S_t are the numbers of infectious particles before and at time t after beginning of inactivation, A_0 is the initial concentration of BPL, k is the initial infectivity inactivation rate constant, and k_1 is the total rate constant for the consumption of BPL during inactivation. This description of inactivation kinetics allows, when rate constants are known, the calculation duration of inactivation necessary to reduce infectivity of the virus to any given extent, which can ensure a completely inactivated, safe vaccine (Budowsky and Zalesskaya 1991). Figure 2.2 depicts the theoretical BPL inactivation curves as calculated by the above formula, as well as experimental data. Initial concentrations of BPL and inactivation temperatures are investigated; in all reactions the tailing phenomenon is visible. In the end, the BPL mediated alkylation or acylation of viral constituents, whether nucleic acids or proteins has resulted in the organic compounds being used in virus inactivation for decades, with two, currently licensed, human BPL-inactivated vaccines on the market and multiple vaccines in development.

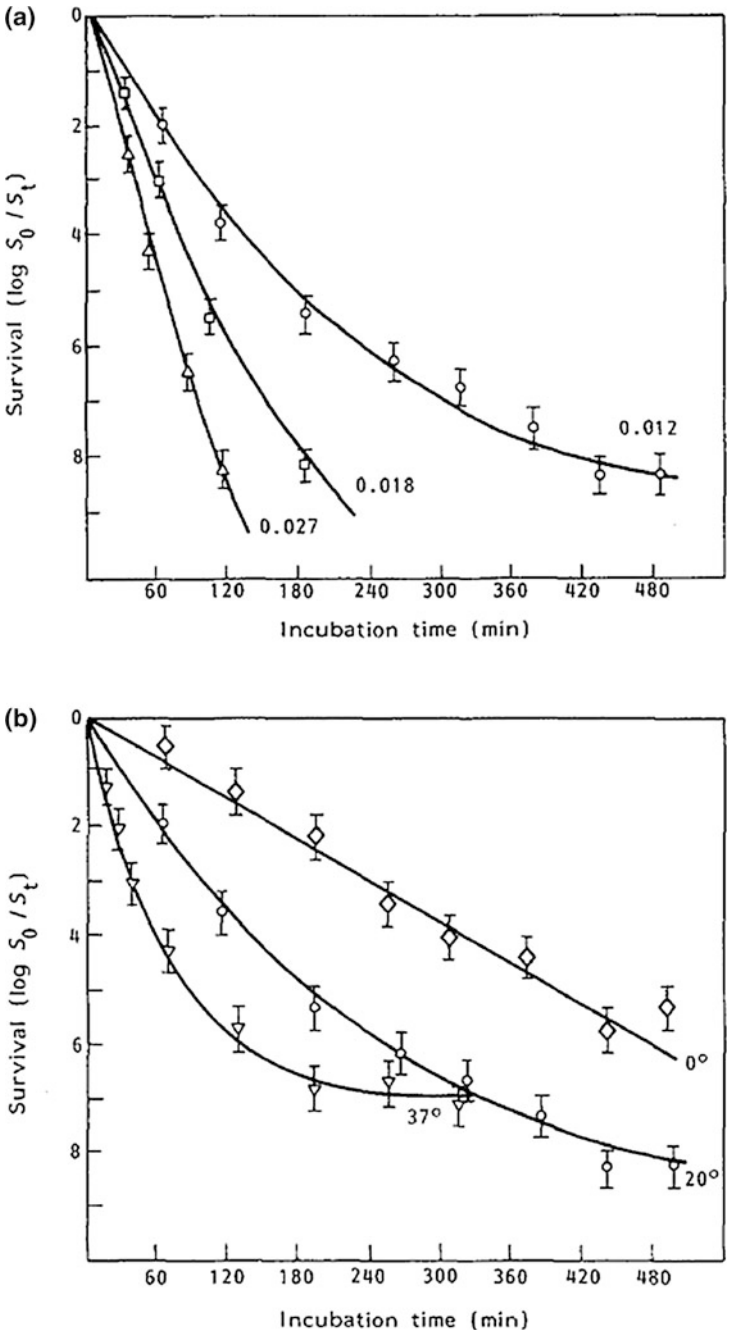


Fig. 2.2 Kinetics of inactivation graph BPL, theoretical curves (*black lines*) calculated according to equation listed above and experimental data (*open diamonds*) of phage MS2 inactivation at different BPL concentrations (**a**) and temperature (**b**). Taken from Budowsky and Zalesskaya 1991, with permission

2.5 Examples of BPL-Inactivated Vaccines

2.5.1 Inactivated Influenza Vaccine

The first reports of vaccination against influenza stem from the 1930s (Stokes et al. 1937) which ultimately lead to the licensure of the first inactivated influenza vaccine in 1945 in the US (Francis et al. 1946; Salk and Francis 1946). Over the course of more than 80 years, the currently available inactivated influenza vaccines have undergone several improvements and have shown significant benefits for society (Clover et al. 1991; Edwards et al. 1994; Gruber et al. 1990; Neuzil et al. 2001; Wilde et al. 1999), however, breadth of protection and efficacy of currently available vaccines are still insufficient to diminish the current annual health burden induced by the virus. Differences in protective efficacy may result from continuing antigenic variation in the prevalent epidemic strains. Due to this variation, the composition of inactivated influenza virus vaccine, unlike that of most viral vaccines, must be kept constantly under review. Accordingly, WHO publishes recommendations concerning the strains to be included in the vaccine twice annually (WHO 2000, 2009a; Ghendon 1991).

Until recently inactivated influenza vaccines consisted of three inactivated viruses; two Influenza A strains and one B strain, however, a new pattern of influenza B circulation has rendered it troublesome to predict the global dominance of one of the two influenza B lineages (Paiva et al. 2013). Therefore, quadrivalent influenza vaccines have been developed to ensure broader protection against Type B influenza viruses as compared to the trivalent vaccines which contained only one Type B influenza strain from one lineage. The licensed quadrivalent inactivated influenza vaccines are formulated in the same way as their trivalent counterparts, however, two influenza B strains, one from the Victoria lineage and one from the Yamagata lineage, are included in the formulation.

After inactivation the vaccine strains are either formulated as virosomes (Herzog et al. 2009), whole inactivated virus (WIV), or detergent-treated "split" vaccines, where the viral envelope is disrupted after inactivation (Wood 1998; Schultz-Cherry and Jones 2010). All the US-licensed inactivated influenza vaccines are split vaccines as "splitting" of the virus is thought to reduce reactogenicity, especially in children (Verma et al. 2012; Nicholson et al. 2003). However, WIV vaccines have been reported to induce stronger immune responses in immunologically naive individuals than split-virus or subunit vaccines (Beyer et al. 1998; Nicholson et al. 1979). Budimir et al. have recently shown that only WIV influenza vaccines, and not split or subunit vaccines, are capable of inducing cross-protection against heterosubtypic challenge due to elicitation of a strong CTL response in mice as whole (BPL) inactivated vaccines are capable of endosomal fusion into the cell cytoplasm (Budimir et al. 2012). The necessity of an influenza vaccine that can elicit cell-mediated immunity and the superiority of WIV vaccines over split vaccine variants has recently been reviewed (Furuya 2012).

After harvesting the (reassorted) vaccine strains, purification steps such as filtration or sucrose gradient centrifugation may be performed before inactivation of the monovalent bulks. Currently, licensed vaccines are inactivated using either BPL or formaldehyde. Although formaldehyde inactivated vaccines are successful in eliciting protection against influenza, it has been reported that formaldehyde interferes with the fusion ability of the inactivated virus particle (Geeraedts et al. 2012) which in turn prohibits an optimal CTL response. This is not observed for BPL-inactivated influenza virus (Budimir et al. 2012). According to the Ph. Eur (2011c), the concentration of BPL and formaldehyde cannot exceed 0.1 % (v/v) and 0.02 %, respectively, during the entire inactivation procedure. Other inactivation parameters, such as time of inactivation and temperature are not specified. As with formalin-inactivated vaccines, a manufacturer-validated inactivation method must be demonstrated to the approving regulatory body. The principles of influenza inactivation have been investigated with respect to initial BPL concentration, temperature, and composition of the solution where it was shown that inactivation of influenza A at 20 °C at BPL concentrations of 0.011 or 0.055 M ranges from 35 to 170 min, depending on whether the virus sample is purified or in harvested allantoic fluid (Budowsky et al. 1991). Testing for effective inactivation is specified by the Ph. Eur. (2011c), and should be performed by inoculation of either embryonated hens' eggs or the cell substrate that was used for vaccine production with a fixed amount of vaccine product, after which the cultures are tested for abscense of heamaglutination, which is a surrogate for infectious virus activity.

After inactivation, antigen is purified to reduce nonviral contaminants and concentrated by various methods such as centrifugation through sucrose gradient, passage over chromatographic column, dialysis, or filtration. Splitting of the virus particles is achieved by addition of a solvent (an ether or detergent) during or before purification which disrupts the viral membrane. Monovalent vaccines are finally combined to form the final vaccines. Table 2.2 gives an overview of the FDA approved inactivated influenza vaccines, all of which are split vaccines.

Table 2.2 Overview of FDA approved inactivated influenza vaccines

Vaccine	Manufacturer	Inactivation agent	Splitting agent
AFLURIA	CSL	BPL	Sodium taurodeoxycholate
Agriflu	Novartis	Formaldehyde	CTAB
Flulaval (Quadrivalent)	ID Biomedical Corporation of Quebec	UV + Formaldehyde	Sodium deoxycholate
Fluarix (Quadrivalent)	GlaxoSmithKline	Formaldehyde	Sodium deoxycholate
Fluvirin	Novartis	BPL	Nonylphenol ethoxylate
Fluzone (Quadrivalent)	Sanofi	Formaldehyde	Octylphenol ethoxoylate

2.5.2 Inactivated Rabies Vaccine

The second currently licensed BPL-inactivated viral vaccine is a rabies vaccine which has an equally rich history of development. Pasteur introduced an experimental rabies vaccine in 1885 when he observed the rapid decrease of rabies virus virulence upon air drying of rabies-infected rabbit spinal cords. Serially less dried rabies-infected rabbit spinal cords containing inactivated—or at least partially inactivated—rabies viruses induced protection of dogs and later humans against challenge following inoculation (Bazin 2011; Pasteur et al. 1885). This method of vaccination, although it was considered a treatment for infected people at the time, was the foundation for rabies vaccines. However, Pasteur faced significant criticism from the scientific community as recipients were essentially inoculated with virulent virus at the end of the treatment (Burke 1996; Gelfand 2002; Wu et al. 2011). This set the incentive to chemically inactivate the rabies virus with phenol in 1908 leading to the first completely inactivated rabies vaccine, despite the disruptive action of phenol on the antigenic sites on the proteins (Fermi 1908; Semple 1911; Briggs 2012).

In the 1950s and 1960s the vaccine was further improved by using alternative substances to cultivate rabies virus, such as chicken and duck embryos (Peck et al. 1955). This due to the fact that vaccines based on adult mammalian nerve tissue were associated with effects such as encephalomyelitis and demyelination lesions in the CNS due to the presence of myelin (Bonito et al. 2004; Bahri et al. 1996). Therefore, the WHO currently does not recommend the use and production of nerve tissue vaccines (WHO 2005) and has been advocating use of cell culture or embryonated eggs as production platforms since 1983 (WHO 1984). In the US, only cell culture derived rabies vaccines are approved for commercial use, however, some African and Latin American countries continue to produce and use nerve tissue vaccines by phenol inactivation, where the vaccine production protocol resembles the methods from a century ago (Briggs 2012). Today, there are two primary avian cell lines used for rabies vaccine production; purified chick embryo cell vaccine (PCECV) and purified duck embryo rabies vaccine (PDEV) and multiple continuous cell lines such as MRC-5, Vero, and primary hamster kidney cells. However, inactivated vaccines produced on continuous cell lines are not completely free from adverse reactions. There are reports on reactogenicity in response to vaccination with the human diploid cell rabies vaccine (HDCRV) which may relate to the presence of BPL-altered human albumin, added as a stabilizer to vaccine preparations (Anderson et al. 1987; Swanson et al. 1987). Nonetheless, cell culture based vaccines are still vastly preferred over nerve tissue vaccines. Moreover, an additional advantage of the use of a cell line platform, for instance Vero cells, is that they can be cultured in large scale in fermenters on microcarriers which contributes to standardization, safety, and upscaling of the production system resulting in constant yields.

Despite the variation in vaccine cell substrates, the majority of the rabies vaccines are inactivated in a similar manner using a concentration of not more than

1:3,500 and up to 1:5,000 v/v of BPL at 2–8 °C for 24 h (WHO 2007a; Ph. Eur. 2011e). However, there are exceptions such as the use of formalin for Primary Hamster kidney cell culture vaccine (PHKCV). As with the formalin-inactivated vaccines, the inactivation curves have to be validated and approved by the regulatory body. After inactivation, different purification standards can be used such as ultrafiltration, ultracentrifugation, zonal centrifugation, or chromatography. Once formulated, the vaccine potency for all these vaccines is determined by quantifying the degrees of protection against rabies following immunizing and intracerebral challenge of mice (de Moura et al. 2009; Fitzgerald et al. 1978). Based on the results of this National Institutes of Health (NIH) test, the vaccine dosing is set at 2.5 International Units/dose. Many regulatory authorities, including the Ph. Eur. and WHO, have adopted the NIH potency test as the only assay for potency quantification of inactivated Rabies vaccines, despite the recognition of the fact that the animal test should be replaced by an antigen quantification procedure (Bruckner et al. 2003). The vaccine is further tested for complete inactivation by inoculating the cell substrate used for manufacturing with 25 human vaccine doses or more. Cultures are examined for the presence of newly produced rabies virus using immunofluorescence.

2.5.3 BPL-Inactivated Vaccines Summarized

As with the formaldehyde inactivated vaccines, BPL-inactivated vaccines have varying inactivation procedures, with different temperatures, BPL concentrations, and inactivation times for complete inactivation. A thoroughly studied inactivation curve of virus activity is required for validation of inactivation parameters and confident declaration of viral absence, which a regulatory agency can consequently approve for vaccine licensure. As compared to formaldehyde inactivation, BPL inactivation times are significantly shorter where minutes to hours can suffice in inactivating viral activity as compared to the days or months needed for formaldehyde inactivation. Further advantages of BPL inactivation is the lower inactivation temperature which may prevent thermal degradation of important epitopes, moreover, protein moieties are less likely to be altered by BPL due to the primary reaction of the compound with nucleic acids. Despite the evident advantages of BPL, formaldehyde is more widely used for inactivation of pathogens, perhaps due to historical use of the compound and years of experience which has paved regulatory pathways for the licensure. Nonetheless, it is evident that the advantages of BPL should be considered by vaccine manufacturers for viral inactivation.

2.6 Inactivated Vaccines in Development

The century old concept of the use of inactivating viruses to elicit protection against the virulent pathogen continues to bear fruit for humanity. Countless improvements and innovations in the field of vaccinology, such as the introduction of recombinant, DNA-based, and vectored vaccines have not stopped the use and development of inactivated vaccines. The relative straightforwardness in which an inactivated vaccine is produced and licensed, accompanied by the fact that inactivated vaccines cannot revert as their replication competent counterparts can do, explains the fact that there are new inactivated pathogens that are being evaluated as vaccine candidates. However, inactivation does not always guarantee the creation of a suitable vaccine as was observed with pathogens such as RSV and measles, therefore immunogenicity of the novel inactivated particle must always be thoroughly tested. Furthermore, new inactivation methods are also being investigated to circumvent the disadvantages of formalin and BPL such as altered immunogenicity due to epitope masking. This section will provide an overview of novel inactivated vaccines in development as well as new inactivation methods.

2.6.1 New Inactivation Methods

The increased safety associated with inactivated vaccines does not entail a spotless track record, as was described for the formalin-inactivated RSV and measles vaccines. The inadequate immune response induced with inactivated viruses is thought to be due to masking of essential epitopes. This drives the investigation of alternative inactivation methods that do not alter epitopes or skew immune responses to ensure a protective vaccine with high efficiency. Three new inactivation methods, being hydrogen peroxide treatment, zinc-finger reactive treatment, and gamma irradiation are described in more detail below. Whether these inactivation methods will be implemented in the manufacturing of vaccines remains to be determined.

A hydrogen peroxide based vaccine platform has been proposed by Amanna et al. (2012). Oxidizing agents are an essential part of the mammalian innate immune system (Valko et al. 2007) and use of such agents, like H_2O_2, as antimicrobial and antiseptic agents have been well established (Linley et al. 2012). However, the use of H_2O_2 in the inactivated vaccine industry was never considered as H_2O_2 is believed to irreversibly damage basic molecular structure of proteins (Skykes 1965). H_2O_2 inactivation of a range of DNA and RNA viruses showed minimal damage to epitopes compared to BPL and formalin. In addition, superiority of H_2O_2 inactivated vaccines was demonstrated by the elicitation of a strong neutralizing antibody response, effective T cell responses, and protection in mice (Amanna et al. 2012). Furthermore, the use of H_2O_2 requires a much shorter inactivation time in comparison to formalin and decomposes into nontoxic products (water and oxygen). Mechanism for H_2O_2 inactivation is the genomic damage induced by hydroxyl

radical attack on nucleosides resulting in single- or double-strand breaks ultimately leading to inactivation of the virus (Termini 2000). The authors propose H_2O_2 as a feasible, broad-spectrum, and effective inactivation platform.

Conserved zinc-finger motifs within the small, basic, nucleic acid-binding nucleocapsid proteins of retroviruses are essential for virus replication (Aldovini and Young 1990). The vital role of this motif has lead to the discovery and development of various compounds that covalently bind these motifs and abrogate infectivity of viruses (Rice et al. 1995). Despite the loss of infectivity, the inactivated virus still retains the capacity to enter target cells as well as its structural and functional integrity (Rossio et al. 1998). To that end, use of zinc-finger reactive compounds was examined for RSV inactivation with maximum preservation of the virion surface structure. RSV contains zinc-finger motifs in the M2-1 protein, necessary for processivity of the viral polymerase (Hardy and Wertz 1998). The 2,2-dithiodipyridine zinc-finger reactive compound was shown to effectively inactivate RSV and conveyed moderate immunogenicity in cotton rats, which could be raised dramatically in combination with the ribi adjuvant system (RAS), an oil-in-water emulsion. However, addition of this adjuvant also induced the enhanced disease, which was not observed with the inactivated RSV alone (Boukhvalova et al. 2010). More research would be necessary to produce a safe and immunogenic-inactivated RSV, however the authors argue the superiority of zinc-finger reactive compounds over the traditional methods with respect to an unaltered protein structure and feasibility of upscaling for large scale production.

Gamma irradiation as a physical means of virus inactivation is not a novel method as it has been used extensively in the past for investigational vaccine purposes (Campbell 1985; Marennikova and Macevic 1975; Reitman and Tonik 1971; Reitman et al. 1970; Wiktor et al. 1972). It is argued that γ-irradiation is superior to the conventional chemical methods (formalin and BPL) due to the view that organisms can be rendered incapable of replication by generating strand breaks in genetic material without structural destruction of proteins (Furuya 2012). Furthermore, γ-irradiation is associated with high penetration capacity allowing viral inactivation of large volumes, stored in closed containers, and even in frozen state. Moreover, there is no need to remove a chemical compound after inactivation (Furuya 2012). Despite all these advantages no γ-irradiated vaccine exists today, this presumably due to the success of formalin and BPL which represent well-established and regulatory-accepted inactivation methods, which manufacturer's may prefer for vaccine production. Furthermore, concerns surrounding the safety of γ-irradiation may also have inhibited its use in the vaccine industry, however, these concerns may diminish as more research on the application of γ-irradiation is increasing (Frenzen et al. 2001). Recently, a γ-irradiated Venezuelan equine encephalitis virus (VEE) strain has shown to convey protection against subcutaneous challenge at low doses in mice, however, despite the hypothesis that γ-irradiation should not affect epitopes, a decrease in antigenicity was observed after inactivation and protection against aerosol challenge was suboptimal (Martin et al. 2010a), nonetheless, this VEE vaccine candidate will be further pursued by the authors, as well as a formalin-inactivated counterpart (Martin et al. 2010b).

Gamma irradiation has also been proposed for whole inactivated influenza where improved heterotypic immunity was observed, primarily mediated by cross reactive T cells (Alsharifi and Mullbacher 2010; Furuya et al. 2010).

2.6.2 New Targets for Whole Inactivated Vaccine Development

2.6.2.1 Inactivation of Attenuated Viruses

As described earlier in the chapter, attenuated vaccine viruses may revert to a virulent form which would make them capable of causing the disease against which they should protect. There are multiple licensed attenuated vaccine viruses that are currently being considered for inactivation for vaccine purposes.

The oral polio vaccine (OPV) displays frequent reversion to virulence in vaccine recipients and there are estimates of approximately 400–800 vaccine-associated paralytic poliomyelitis (VAPP) cases per year globally (John 2002). Despite the immediate recognition of the fact that OPV strains can revert readily into a pathogenic phenotype (Henderson et al. 1964), OPV has been used since the 1960s and still is being used extensively. However, recently, it has been acknowledged that use of the oral live attenuated vaccine is at odds with global eradication of poliomyelitis. Indeed, the number of vaccine-associated poliomyelitis cases is in the range of wild-type PV induced poliomyelitis cases (WHO 2006a). Although IPV is a safe alternative, the costs of currently available IPV are too high to implement its use in low income countries (Heinsbroek and Ruitenberg 2010; Zehrung 2010) and several options to reduce costs of IPV are being considered (WHO 2009b). In the era after eradication, IPV use will have to be continued at least for a certain amount of time. At that time, production of IPV from wild-type PV strains will fall under strict biosafety measures (WHO 2009c). Even though it is currently not clear whether an IPV based on OPV strains may be produced at lower biosafety level after eradication as compared to a wild-type based IPV, there is much research going on to the inactivation of the OPV strains with formalin to eventually replace the inactivated PV vaccine based on the wild-type strains. Not only would the lowering of biosafety level decrease potential costs of goods, replacing the wild-type strains greatly reduces the risks of poliomyelitis upon accidental outbreaks from the manufacturing facility, after eradication. The manufacture of Sabin-IPV is essentially identical to the Salk-IPV process with slight modifications (Westdijk et al. 2011). The WHO encourages the development of this Sabin-IPV vaccine (Bakker et al. 2011) and multiple clinical trials have been or are being performed (Verdijk et al. 2011), moreover, in Japan a Sabin based IPV has recently been licensed in combination with diphtheria, tetanus, and acellular pertussis (DTaP-Sabin IPV) (Mahmood et al. 2013). In general, Sabin-IPV displays higher immunogenicity for serotype 1, lower for type 2, and similar for type 3 in comparison to Salk-IPV, licensure of more Sabin derived IPV's is foreseen in the near future.

Monath et al. describe the results of a Phase I study of a BPL-inactivated Yellow Fever (YF) vaccine, based on the licensed attenuated 17D strain (Monath et al. 2011). The 17D vaccine was developed in 1936 by Max Theiler and today 20 million doses are issued per year. However, yellow fever vaccine-associated viscerotropic disease (YF-AVD) and yellow fever vaccine-associated neurological disease (YF-AND) occurring at a frequency of 0.4 and 1.8 per 100,000 doses, respectively (Lindsey et al. 2008), instigate a need for safer vaccines. Inactivated vaccines will reduce the adverse effects associated with the vaccine and is predicted to be less reactogenic as it has been cultivated on Vero cells instead of eggs (Hayes 2010). The alum-adjuvanted, BPL-inactivated vaccine induced neutralizing antibodies in a high percentage of subjects, albeit lower titers than the live vaccine, whether the lower titers will be compensated for by the higher safety profile is yet to be determined (Monath et al. 2011).

The oral rotavirus vaccines have also been shown to be disadvantageous with respect to safety and regional inefficacy to induce an adequate immune response, consequently, an inactivated counterpart has been proposed for development (Jiang et al. 2008a). As "conventional" chemical inactivation of rotavirus with formaldehyde or BPL treatment has shown to destroy integrity of the immunogenic epitopes (Offit and Dudzik 1989; Zissis et al. 1983), a novel heat inactivated rotavirus vaccine (IRV) has been proposed for development. Rotavirus inactivated at 60 °C for 2 h has shown to be immunogenic in mice (Jiang et al. 2008b) and gnotobiotic piglets when administered together with an aluminum adjuvant (Wang et al. 2010).

Another example of inactivation of attenuated virus strains for vaccine purposes is the inactivation of the attenuated Varicella-Zoster Virus (VZV) Oka/Merck strain. The live attenuated vaccine provides protection against varicella (chicken pox) and zoster (shingles), however, despite the high safety profile (Galea et al. 2008), there was stimulus to inactivate this attenuated strain for use in prevention of zoster in immunocompromised patients (Hata et al. 2002; Redman et al. 1997). Currently Phase III clinical trials are ongoing for this heat inactivated vaccine which has been reported to be (almost completely) inactivated by heating at 56 °C for 7 days which reduced the pfu/ml of the live vaccine preparation from 4,000 to 2 pfu/ml in the "inactivated" vaccine (Levine et al. 2000).

2.6.2.2 Inactivation of Wild-Type Viruses

The successes of the current inactivated viral vaccines have stimulated the research and development of other virus inactivated vaccines using formalin or BPL. A selection of interesting candidate vaccines is listed below.

Enterovirus 71 (EV71) is a picornavirus capable of inducing hand, foot, and mouth disease (HFMD) which can lead to serious neurological complications and even death (McMinn 2002). As with the other picornaviridea vaccines (PV and HAV) formalin was chosen as inactivating agent to develop an inactivated EV71 vaccine, propagated on Vero cells. In Taiwan, a pilot production procedure has been developed resulting in an optimized USP and DSP procedure. Inactivation studies

demonstrated that inactivation of the entire vaccine bulk would require 2.2, 12.6, and 31.6 days at 37, 25, and 4 °C, respectively (Chong et al. 2012). A Phase I clinical study has been scheduled using this inactivated bulk. In China, there are three Phase III clinical studies ongoing with a formalin-inactivated EV71 C4 strain grown on either Vero cells (Li et al. 2012; Zhu et al. 2013) or on human diploid KMB-17 cells (Dong et al. 2011), all adsorbed to aluminum hydroxide as an adjuvant.

The inactivated flavivirus vaccines against JEV and TBEV, have encouraged researchers to examine formalin inactivation of another member of the flavivirus family, namely Dengue viruses. A cell culture adapted Dengue virus type 2 (DENV2) that was propagated on Vero cells was inactivated with 0.05 % formalin for 10 days at 22 °C and adsorbed to alum hydroxide. This candidate elicited neutralizing antibodies as well as protection in macaques (Putnak et al. 2005). However, this monovalent candidate has not been further pursued since its development as it has been recognized that Dengue requires a potent vaccine that can elicit equally protective immune response against all 4 serotypes without risks of waning immunity (Heinz and Stiasny 2012).

Mosquito-transmitted *alphaviruses* such as Venezuelan, Western and Eastern equine encephalitis virus (VEE, WEE, and EEE virus) have been formalin-inactivated for use as vaccines for horses (Zacks and Paessler 2009). However, there are no commercially available *alphavirus* vaccines licensed for human use despite their development and use as investigational vaccines against bioterrorist threats by the US army. The reason for halted development most likely lies in the only moderate immunogenicity of this candidate (Edelman et al. 1979; Steele et al. 2007). Research focusing on the less known *alphavirus* Ross River virus (RRV), which is endemic in Australia and Papua New Guinea, causing epidemic polyarthritis (Harley et al. 2001), has lead to a phase 1/2 dose escalation study using an adjuvanted, formalin-inactivated RRV vaccine which showed high seroconversion rates in naïve healthy young adults (Aichinger et al. 2011). Another inactivated *alphavirus* vaccine in development is a chikungunya virus (CHIKV) inactivated vaccine, which has been studied in mice. Formalin, as well as BPL, inactivated CHIKV vaccines in combination with three different adjuvants were compared. The alum-adjuvanted, BPL-inactivated vaccine induced highest antibody titers, however, all inactivated CHIKV vaccine formulations conveyed some degree of reduction in viral replication after homologous challenge (Kumar et al. 2012).

Inactivation of respiratory syncytial virus (RSV) with formalin in the 1960s resulted in hospitalization and even deaths of infants as described earlier in this chapter. Therefore, inactivation was deemed an unsuitable and even dangerous method for RSV vaccine development. Nonetheless, a BPL-inactivated RSV vaccine supplemented with toll-like receptor (TLR) ligands and with total respiratory tract (TRT) administration has recently been proposed in mice as a nonreplicating RSV vaccine without the enhanced disease due to proper immune stimulation (Shafique et al. 2012). Inactivation was achieved by a 0.025 % BPL concentration for 16 h at 4 °C. Administration of this vaccine in combination with innate receptor ligands, induced a protective Th1 immune response. In addition, the protection was achieved without priming for enhanced disease.

Another example of a novel BPL-inactivated pathogen is the Severe Acute Respiratory Syndrome (SARS) Corona Virus propagated on Vero cells. When immunized in combination with an adjuvant mice and golden Syrian hamsters conveyed the induction of high titer neutralizing antibodies and protection from challenge (Roberts et al. 2010).

2.7 Concluding Remarks

Time will tell whether one or more of these vaccines in development will contribute to the struggle against infectious diseases in the form of a licensed inactivated vaccine. The extensive knowledge, and experience achieved with the existing viral inactivation procedures for vaccine manufacture will continue to serve as a foundation of vaccinology for novel inactivated vaccines. Today millions of people are, and will be, protected worldwide with inactivated viral vaccines. Furthermore, this number will presumably continue to grow as research in novel inactivated vaccines expands, as this method of vaccine preparation remains a relatively straightforward way to produce safe and effective vaccines. The concept of virus inactivation for vaccine production can be therefore seen as the low-hanging fruit within the tree of vaccine design, bearing in mind that complete understanding of viral inactivation and immunogenicity of the resulting particle are essential for success.

References

Aichinger G, Ehrlich HJ, Aaskov JG, Fritsch S, Thomasser C, Draxler W, Wolzt M, Muller M, Pinl F, Van Damme P, Hens A, Levy J, Portsmouth D, Holzer G, Kistner O, Kreil TR, Barrett PN (2011) Safety and immunogenicity of an inactivated whole virus Vero cell-derived Ross River virus vaccine: a randomized trial. Vaccine 29(50):9376–9384

Aldovini A, Young RA (1990) Mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging result in production of noninfectious virus. J Virol 64(5):1920–1926

Alsharifi M, Mullbacher A (2010) The gamma-irradiated influenza vaccine and the prospect of producing safe vaccines in general. Immunol Cell Biol 88(2):103–104

Amanna IJ, Raue HP, Slifka MK (2012) Development of a new hydrogen peroxide-based vaccine platform. Nat Med 18(6):974–979

Anderson MC, Baer H, Frazier DJ, Quinnan GV (1987) The role of specific IgE and beta-propiolactone in reactions resulting from booster doses of human diploid cell rabies vaccine. J Allergy Clin Immunol 80(6):861–868

Andre FE (1995) Approaches to a vaccine against hepatitis A: development and manufacture of an inactivated vaccine. J Infect Dis 171(Suppl 1):S33–S39

Annunziato D, Kaplan MH, Hall WW, Ichinose H, Lin JH, Balsam D, Paladino VS (1982) Atypical measles syndrome: pathologic and serologic findings. Pediatrics 70(2):203–209

Armstrong ME, Giesa PA, Davide JP, Redner F, Waterbury JA, Rhoad AE, Keys RD, Provost PJ, Lewis JA (1993) Development of the formalin-inactivated hepatitis A vaccine, VAQTA from the live attenuated virus strain CR326F. J Hepatol 18(Suppl 2):S20–S26

Bahnemann HG (1990) Inactivation of viral antigens for vaccine preparation with particular reference to the application of binary ethylenimine. Vaccine 8(4):299–303

Bahri F, Letaief A, Ernez M, Elouni J, Chekir T, Ben Ammou S, Jemni L (1996) Neurological complications in adults following rabies vaccine prepared from animal brains. Presse Med 25 (10):491–493

Bakker WA, Thomassen YE, van't Oever AG, Westdijk J, van Oijen MG, Sundermann LC, van't Veld P, Sleeman E, van Nimwegen FW, Hamidi A, Kersten GF, van den Heuvel N, Hendriks JT, van der Pol LA (2011) Inactivated polio vaccine development for technology transfer using attenuated Sabin poliovirus strains to shift from Salk-IPV to Sabin-IPV. Vaccine 29 (41):7188–7196

Barrett PN, Mundt W, Kistner O, Howard MK (2009) Vero cell platform in vaccine production: moving towards cell culture-based viral vaccines. Expert Rev Vaccines 8(5):607–618

Bazin H (2011) Pasteur and the birth of vaccines made in the laboratory. In: Plotkin SA (ed) History of vaccine development. Springer, New York, pp 39–44. doi:10.1007/978-1-4419-1339-5_6

Beale AJ (1961) The D-antigen content in poliovaccine as a measure of potency. Lancet 2 (7213):1166–1168

Beardmore WB, Hook AE, Sarber RW, Mc LI Jr, Taylor AR (1957) Poliomyelitis vaccine safety testing by the tissue culture method. J Immunol 79(6):489–496

Beyer WE, Palache AM, Osterhaus AD (1998) Comparison of serology and reactogenicity between influenza subunit vaccines and whole virus or split vaccines: a review and meta-analysis of the literature. Clin Drug Invest 15(1):1–12

Bodian D (1958) Control of the manufacture of poliomyelitis vaccine. In: Poliomyelitis—papers and discussions presented at the fourth international poliomyelitis conference, pp 77–85

Bonito RF, de Oliveira NM, Nishioka Sde A (2004) Adverse reactions associated with a Fuenzalida-Palacios rabies vaccine: a quasi-experimental study. Rev Soc Bras Med Trop 37 (1):7–9

Bottiger M, Lycke E, Melen B, Wrange G (1958) Inactivation of poliomyelitis virus by formaldehyde; incubation time in tissue culture of formalin treated virus. Arch Gesamte Virusforsch 8(2):259–266

Boukhvalova MS, Prince GA, Blanco JC (2010) Inactivation of respiratory syncytial virus by zinc finger reactive compounds. Virol J 7:20

Bovier PA (2008) Epaxal: a virosomal vaccine to prevent hepatitis A infection. Expert Rev Vaccines 7(8):1141–1150

Briggs DJ (2012) The role of vaccination in rabies prevention. Curr Opin Virol 2(3):309–314

Brodie M, Park WH (1936) Active immunization against poliomyelitis. Am J Public Health Nations Health 26(2):119–125

Brown F (1993) Review of accidents caused by incomplete inactivation of viruses. Dev Biol Stand 81:103–107

Brown F (1995) Formaldehyde as an inactivant. Vaccine 13(2):231

Bruckner L, Cussler K, Halder M, Barrat J, Castle P, Duchow K, Gatewood DM, Gibert R, Groen J, Knapp B, Levis R, Milne C, Parker S, Stunkel K, Visser N, Volkers P (2003) Three Rs approaches in the quality control of inactivated rabies vaccines. The report and recommendations of ECVAM workshop 48. Altern Lab Anim 31(4):429–454

Budimir N, Huckriede A, Meijerhof T, Boon L, Gostick E, Price DA, Wilschut J, de Haan A (2012) Induction of heterosubtypic cross-protection against influenza by a whole inactivated virus vaccine: the role of viral membrane fusion activity. PLoS ONE 7(1):e30898

Budowsky EI, Bresler SE, Friedman EA, Zheleznova NV (1981) Principles of selective inactivation of viral genome. I. UV-induced inactivation of influenza virus. Arch Virol 68 (3–4):239–247

Budowsky EI, Friedman EA, Zheleznova NV, Noskov FS (1991) Principles of selective inactivation of viral genome. VI. Inactivation of the infectivity of the influenza virus by the action of beta-propiolactone. Vaccine 9(6):398–402

Budowsky EI, Zalesskaya MA (1991) Principles of selective inactivation of viral genome. V. Rational selection of conditions for inactivation of the viral suspension infectivity to a given extent by the action of beta-propiolactone. Vaccine 9(5):319–325

Bungener L, Huckriede A, de Mare A, de Vries-Idema J, Wilschut J, Daemen T (2005) Virosome-mediated delivery of protein antigens in vivo: efficient induction of class I MHC-restricted cytotoxic T lymphocyte activity. Vaccine 23(10):1232–1241

Burke DS (1996) Joseph-Alexandre Auzias-Turenne, Louis Pasteur, and early concepts of virulence, attenuation, and vaccination. Perspect Biol Med 39(2):171–186

Campbell CH (1985) Immunogenicity of bluetongue virus inactivated by gamma irradiation. Vaccine 3(5):401–406

Castilow EM, Olson MR, Varga SM (2007) Understanding respiratory syncytial virus (RSV) vaccine-enhanced disease. Immunol Res 39(1–3):225–239

Chandler MD, Frerichs GN (1980) Effect of metabisulphite on residual free formaldehyde estimations. J Biol Stand 8(2):145–149

Charney J, Fisher WP, Machlowitz RA (1957) Preparation and inactivation of purified poliovirus: comparison of vaccines derived from Mahoney and Parker poliovirus. Proc Soc Exp Biol Med 96(3):601–605

Chong P, Hsieh SY, Liu CC, Chou AH, Chang JY, Wu SC, Liu SJ, Chow YH, Su IJ, Klein M (2012) Production of EV71 vaccine candidates. Hum Vaccin Immunother 8(12):1775–1783

Clover RD, Crawford S, Glezen WP, Taber LH, Matson CC, Couch RB (1991) Comparison of heterotypic protection against influenza A/Taiwan/86 (H1N1) by attenuated and inactivated vaccines to A/Chile/83-like viruses. J Infect Dis 163(2):300–304

Colburn NH, Richardson RG, Boutwell RK (1965) Studies of the reaction of beta-propiolactone with deoxyguanosine and related compounds. Biochem Pharmacol 14(7):1113–1118

Cornfield J, Halperin M, Moore F (1956) Some statistical aspects of safety testing the Salk poliomyelitis vaccine. Public Health Rep 71(10):1045–1056

de Moura WC, de Araujo HP, Cabello PH, Romijn PC, Leite JP (2009) Potency evaluation of rabies vaccine for human use: the impact of the reduction in the number of animals per dilution. J Virol Methods 158(1–2):84–92

Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP, Diaz L, Trento A, Chang HY, Mitzner W, Ravetch J, Melero JA, Irusta PM, Polack FP (2009) Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nat Med 15(1):34–41

Di Trani L, Cordioli P, Falcone E, Lombardi G, Moreno A, Sala G, Tollis M (2003) Standardization of an inactivated H17N1 avian influenza vaccine and efficacy against A/Chicken/Italy/13474/99 high-pathogenicity virus infection. Avian Dis 47(3 Suppl):1042–1046

Dijkstra J (1975) In vitro reaction of beta-propiolactone and gamma-butyrolactone with glutathione and cysteine. Chem Biol Interact 10(2):115–121

Dong C, Liu L, Zhao H, Wang J, Liao Y, Zhang X, Na R, Liang Y, Wang L, Li Q (2011) Immunoprotection elicited by an enterovirus type 71 experimental inactivated vaccine in mice and rhesus monkeys. Vaccine 29(37):6269–6275

Dulbecco R, Vogt M (1954) Plaque formation and isolation of pure lines with poliomyelitis viruses. J Exp Med 99(2):167–182

Edelman R, Ascher MS, Oster CN, Ramsburg HH, Cole FE, Eddy GA (1979) Evaluation in humans of a new, inactivated vaccine for Venezuelan equine encephalitis virus (C-84). J Infect Dis 140(5):708–715

Edwards KM, Dupont WD, Westrich MK, Plummer WD Jr, Palmer PS, Wright PF (1994) A randomized controlled trial of cold-adapted and inactivated vaccines for the prevention of influenza A disease. J Infect Dis 169(1):68–76

Enders JF, Weller TH, Robbins FC (1949) Cultivation of the lansing strain of poliomyelitis virus in cultures of various human embryonic tissues. Science 109(2822):85–87

Farrell LN, Wood W, Macmorine HG, Shimada FT, Graham DG (1955) Preparation of poliomyelitis virus for production of vaccine for the 1954 field trial. Can J Public Health 46 (7):265–272

Fermi C (1908) Uber die Immunisierung gegen Wutkranheit. Z Hyg Infectionskrankh 58:233–276
Fitzgerald EA, Gallagher M, Hunter WS, Seligmann EB Jr (1978) Use of the antibody assay in immunized mice for the determination of rabies vaccine potency. Dev Biol Stand 40:183–186
Francis T Jr, Salk JE, Brace WM (1946) The protective effect of vaccination against epidemic influenza B. J Am Med Assoc 131:275–278
Frenzen PD, DeBess EE, Hechemy KE, Kassenborg H, Kennedy M, McCombs K, McNees A (2001) Consumer acceptance of irradiated meat and poultry in the United States. J Food Prot 64 (12):2020–2026
Frerichs GN, Chandler MD (1980) Estimation of residual free formaldehyde in biological products. J Biol Stand 8(2):139–144
Furuya Y (2012) Return of inactivated whole-virus vaccine for superior efficacy. Immunol Cell Biol 90(6):571–578
Furuya Y, Chan J, Regner M, Lobigs M, Koskinen A, Kok T, Manavis J, Li P, Mullbacher A, Alsharifi M (2010) Cytotoxic T cells are the predominant players providing cross-protective immunity induced by {gamma}-irradiated influenza A viruses. J Virol 84(9):4212–4221
Galea SA, Sweet A, Beninger P, Steinberg SP, Larussa PS, Gershon AA, Sharrar RG (2008) The safety profile of varicella vaccine: a 10-year review. J Infect Dis 197(Suppl 2):S165–S169
Gard S (1957a) Chemical inactivation of viruses. In: Wolstenholme GEW, Millar ECP (eds) Ciba foundation symposium-the nature of viruses, John Wiley & Sons Ltd., Chichester, UK. doi:10.1002/9780470715239.ch8
Gard S (1957b) Inactivation of poliovirus by formaldehyde: theoretical and practical aspects. Bull World Health Organ 17(6):979–989
Gard S, Lycke E (1957) Inactivation of poliovirus by formaldehyde; analysis of inactivation curves. Arch Gesamte Virusforsch 7(5):471–482
Gard S, Lycke E, Olin G, Wesslen T (1957) Inactivation of poliomyelitis virus by formaldehyde. Arch Gesamte Virusforsch 7(2):125–135
Geeraedts F, Ter Veer W, Wilschut J, Huckriede A, de Haan A (2012) Effect of viral membrane fusion activity on antibody induction by influenza H5N1 whole inactivated virus vaccine. Vaccine 30(45):6501–6507
Gelfand T (2002) 11 January 1887, the day medicine changed: Joseph Grancher's defense of Pasteur's treatment for rabies. Bull Hist Med 76(4):698–718
Ghendon Y (1991) Influenza surveillance. Bull World Health Organ 69(5):509–515
Glenny AT, Hopkins BE (1923) Diphtheria toxoid as an immunising agent. Br J Exp Pathol 4:283–288
Gluck R, Mischler R, Brantschen S, Just M, Althaus B, Cryz SJ Jr (1992) Immunopotentiating reconstituted influenza virus virosome vaccine delivery system for immunization against hepatitis A. J Clin Invest 90(6):2491–2495
Griffin DE, Pan CH (2009) Measles: old vaccines, new vaccines. Curr Top Microbiol Immunol 330:191–212
Gruber WC, Taber LH, Glezen WP, Clover RD, Abell TD, Demmler RW, Couch RB (1990) Live attenuated and inactivated influenza vaccine in school-age children. Am J Dis Child 144 (5):595–600
Gupta RK (1998) Aluminum compounds as vaccine adjuvants. Adv Drug Deliv Rev 32(3):155–172
Haffkine WM (1899) Protective inoculation against plague and cholera. BMJ 1:35–36
Halstead SB, Tsai TF (2004) Japanese encephalitis vaccines. In: Bell BP, Feinstone SM (eds) Vaccines, 4th edn. Saunders Elsevier, Philadelphia
Hardy RW, Wertz GW (1998) The product of the respiratory syncytial virus M2 gene ORF1 enhances readthrough of intergenic junctions during viral transcription. J Virol 72(1):520–526
Harley D, Sleigh A, Ritchie S (2001) Ross River virus transmission, infection, and disease: a cross-disciplinary review. Clin Microbiol Rev 14(4):909–932
Hartman FW, Logrippo G, Kelly AR (1954) Preparation and sterilization of blood plasma. Am J Clin Pathol 24(3):339–348
Hartman FW, Logrippo GA (1957) Betapropiolactone in sterilization of vaccines, tissue grafts, and plasma. J Am Med Assoc 164(3):258–260

Hata A, Asanuma H, Rinki M, Sharp M, Wong RM, Blume K, Arvin AM (2002) Use of an inactivated varicella vaccine in recipients of hematopoietic-cell transplants. N Engl J Med 347 (1):26–34

Hayes EB (2010) Is it time for a new yellow fever vaccine? Vaccine 28(51):8073–8076

Heinsbroek E, Ruitenberg EJ (2010) The global introduction of inactivated polio vaccine can circumvent the oral polio vaccine paradox. Vaccine 28(22):3778–3783

Heinz FX, Stiasny K (2012) Flaviviruses and flavivirus vaccines. Vaccine 30(29):4301–4306

Hemminki K (1981) Reactions of beta-propiolactone, beta-butyrolactone and gamma-butyrolactone with nucleic acids. Chem Biol Interact 34(3):323–331

Henderson DA, Witte JJ, Morris L, Langmuir AD (1964) Paralytic disease associated with oral polio vaccines. JAMA 190:41–48

Herzog C, Hartmann K, Kunzi V, Kursteiner O, Mischler R, Lazar H, Gluck R (2009) Eleven years of Inflexal V-a virosomal adjuvanted influenza vaccine. Vaccine 27(33):4381–4387

Hess RD, Weber F, Watson K, Schmitt S (2012) Regulatory, biosafety and safety challenges for novel cells as substrates for human vaccines. Vaccine 30(17):2715–2727

Hueper WC (1963) Environmental carcinogenesis in man and animals. Ann NY Acad Sci 108:963–1038

Jiang B, Gentsch JR, Glass RI (2008a) Inactivated rotavirus vaccines: a priority for accelerated vaccine development. Vaccine 26(52):6754–6758

Jiang B, Wang Y, Saluzzo JF, Bargeron K, Frachette MJ, Glass RI (2008b) Immunogenicity of a thermally inactivated rotavirus vaccine in mice. Hum Vaccin 4(2):143–147

John TJ (2002) Vaccine-associated paralytic polio in India. Bull World Health Organ 80(11):917

Johnson TR, Graham BS (2004) Contribution of respiratory syncytial virus G antigenicity to vaccine-enhanced illness and the implications for severe disease during primary respiratory syncytial virus infection. Pediatr Infect Dis J 23(1 Suppl):S46–S57

Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE (1969) An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am J Epidemiol 89(4):405–421

Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, Parrott RH (1969) Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol 89(4):422–434

Kumar M, Sudeep AB, Arankalle VA (2012) Evaluation of recombinant E2 protein-based and whole-virus inactivated candidate vaccines against chikungunya virus. Vaccine 30 (43):6142–6149

Larghi OP, Nebel AE (1980) Rabies virus inactivation by binary ethylenimine: new method for inactivated vaccine production. J Clin Microbiol 11(2):120–122

Lawrence SA (1999) Beta-propiolactone and aziridine: their applications in organic synthesis and viral inactivation. Chim Oggi 17:51–54

Lawrence SA (2000) beta-Propiolactone: viral inactivation in vaccines and plasma products. PDA J Pharm Sci Technol 54(3):209–217

Levine MJ, Ellison MC, Zerbe GO, Barber D, Chan C, Stinson D, Jones M, Hayward AR (2000) Comparison of a live attenuated and an inactivated varicella vaccine to boost the varicella-specific immune response in seropositive people 55 years of age and older. Vaccine 18 (25):2915–2920

Li YP, Liang ZL, Gao Q, Huang LR, Mao QY, Wen SQ, Liu Y, Yin WD, Li RC, Wang JZ (2012) Safety and immunogenicity of a novel human Enterovirus 71 (EV71) vaccine: a randomized, placebo-controlled, double-blind, Phase I clinical trial. Vaccine 30(22):3295–3303

Lindsey NP, Schroeder BA, Miller ER, Braun MM, Hinckley AF, Marano N, Slade BA, Barnett ED, Brunette GW, Horan K, Staples JE, Kozarsky PE, Hayes EB (2008) Adverse event reports following yellow fever vaccination. Vaccine 26(48):6077–6082

Linley E, Denyer SP, McDonnell G, Simons C, Maillard JY (2012) Use of hydrogen peroxide as a biocide: new consideration of its mechanisms of biocidal action. J Antimicrob Chemother 67 (7):1589–1596

Ljungman P (2012) Vaccination of immunocompromised patients. Clin Microbiol Infect 18(Suppl 5):93–99
Logrippo GA (1960) Investigations of the use of beta-propiolactone in virus inactivation. Ann NY Acad Sci 83:578–594
Lycke E, Melen B, Wrange G (1957) Studies of the inactivation of poliomyelitis virus by formaldehyde. Arch Gesamte Virusforsch 7(4):378–383
Madhusudana SN, Shamsundar R, Seetharaman S (2004) In vitro inactivation of the rabies virus by ascorbic acid. Int J Infect Dis 8(1):21–25
Mahmood K, Pelkowski S, Atherly D, Sitrin R, Donnelly JJ (2013) Hexavalent IPV-based combination vaccines for public-sector markets of low-resource countries: a product review. Hum Vaccin Immunother 9(9):1894–1902
Marennikova SS, Macevic GR (1975) Experimental study of the role of inactivated vaccine in two-step vaccination against smallpox. Bull World Health Organ 52(1):51–56
Martin SS, Bakken RR, Lind CM, Garcia P, Jenkins E, Glass PJ, Parker MD, Hart MK, Fine DL (2010a) Comparison of the immunological responses and efficacy of gamma-irradiated V3526 vaccine formulations against subcutaneous and aerosol challenge with Venezuelan equine encephalitis virus subtype IAB. Vaccine 28(4):1031–1040
Martin SS, Bakken RR, Lind CM, Garcia P, Jenkins E, Glass PJ, Parker MD, Hart MK, Fine DL (2010b) Evaluation of formalin inactivated V3526 virus with adjuvant as a next generation vaccine candidate for Venezuelan equine encephalitis virus. Vaccine 28(18):3143–3151
Mate U, Solomon JJ, Segal A (1977) In vitro binding of beta-propiolactone to calf thymus DNA and mouse liver DNA to form 1-(2-carboxyethyl) adenine. Chem Biol Interact 18(3):327–336
Maves RC, Ore RM, Porter KR, Kochel TJ (2011) Immunogenicity and protective efficacy of a psoralen-inactivated dengue-1 virus vaccine candidate in Aotus nancymaae monkeys. Vaccine 29(15):2691–2696
McMinn PC (2002) An overview of the evolution of enterovirus 71 and its clinical and public health significance. FEMS Microbiol Rev 26(1):91–107
Meier P (1957) Safety testing of poliomyelitis vaccine. Science 125(3257):1067–1071
Melnick JL (1991) Virus inactivation: lessons from the past. Dev Biol Stand 75:29–36
Metz B, Kersten GF, Baart GJ, de Jong A, Meiring H, ten Hove J, van Steenbergen MJ, Hennink WE, Crommelin DJ, Jiskoot W (2006) Identification of formaldehyde-induced modifications in proteins: reactions with insulin. Bioconjug Chem 17(3):815–822
Metz B, Kersten GF, Hoogerhout P, Brugghe HF, Timmermans HA, de Jong A, Meiring H, ten Hove J, Hennink WE, Crommelin DJ, Jiskoot W (2004) Identification of formaldehyde-induced modifications in proteins: reactions with model peptides. J Biol Chem 279 (8):6235–6243
Midthun K, Ellerbeck E, Gershman K, Calandra G, Krah D, McCaughtry M, Nalin D, Provost P (1991) Safety and immunogenicity of a live attenuated hepatitis A virus vaccine in seronegative volunteers. J Infect Dis 163(4):735–739
Moghaddam A, Olszewska W, Wang B, Tregoning JS, Helson R, Sattentau QJ, Openshaw PJ (2006) A potential molecular mechanism for hypersensitivity caused by formalin-inactivated vaccines. Nat Med 12(8):905–907
Monath TP, Fowler E, Johnson CT, Balser J, Morin MJ, Sisti M, Trent DW (2011) An inactivated cell-culture vaccine against yellow fever. N Engl J Med 364(14):1326–1333
Morgeaux S, Milne C, Daas A (2005) Feasibility study to develop a common in vitro D antigen assay for inactivated poliomyelitis vaccines. Pharmeuropa Bio 2005(1):19–26
Murphy BR, Walsh EE (1988) Formalin-inactivated respiratory syncytial virus vaccine induces antibodies to the fusion glycoprotein that are deficient in fusion-inhibiting activity. J Clin Microbiol 26(8):1595–1597
Nathanson N, Langmuir AD (1963) The cutter incident. poliomyelitis following formaldehyde-inactivated poliovirus vaccination in the United States during the Spring of 1955. II. Relationship of poliomyelitis to cutter vaccine. Am J Hyg 78:29–60

Neuzil KM, Dupont WD, Wright PF, Edwards KM (2001) Efficacy of inactivated and cold-adapted vaccines against influenza A infection, 1985 to 1990: the pediatric experience. Pediatr Infect Dis J 20(8):733–740

Nicholson KG, Tyrrell DA, Harrison P, Potter CW, Jennings R, Clark A, Schild GC, Wood JM, Yetts R, Seagroatt V, Huggins A, Anderson SG (1979) Clinical studies of monovalent inactivated whole virus and subunit A/USSR/77 (H1N1) vaccine: serological responses and clinical reactions. J Biol Stand 7(2):123–136

Nicholson KG, Wood JM, Zambon M (2003) Influenza. Lancet 362(9397):1733–1745

Nims RW, Plavsic M (2012) Polyomavirus inactivation—A review. Biologicals 41(2):63–70

Offit PA (2005) The cutter incident, 50 years later. N Engl J Med 352(14):1411–1412

Offit PA, Dudzik KI (1989) Noninfectious rotavirus (strain RRV) induces an immune response in mice which protects against rotavirus challenge. J Clin Microbiol 27(5):885–888

Ozaki Y, Melnick JL (1963) Reaction of poliovirus and formaldehyde in magnesium chloride solution to enhance potency of killed-virus vaccine. J Immunol 90:429–437

Paiva TM, Benega MA, Silva DB, Santos KC, Cruz AS, Hortenci MF, Barbieri MT, Monteiro MM, Barbosa HA, Carvalhanas TR (2013) Evolutionary pattern of reemerging influenza B/Victoria lineage viruses in Sao Paulo, Brazil, 1996–2012: implications for vaccine composition strategy. J Med Virol 85(11):1983–1989

Pasteur L, Chamberland CE, Roux E (1885) Methode pour prevenir la rage après morsue. C R Acad Sci (101):765–774 (in French)

Patel MM, Haber P, Baggs J, Zuber P, Bines JE, Parashar UD (2009) Intussusception and rotavirus vaccination: a review of the available evidence. Expert Rev Vaccines 8(11):1555–1564

Peck FB Jr, Powell HM, Culbertson CG (1955) A new antirabies vaccine for human use; clinical and laboratory results using rabies vaccine made from embryonated duck eggs. J Lab Clin Med 45(5):679–683

Perrin P, Morgeaux S (1995) Inactivation of DNA by beta-propiolactone. Biologicals 23 (3):207–211

Ph. Eur. (2011a) Free formaldehyde. In: Pharmacopoeia E (ed) European Pharmacopoeia, 7th edn, vol 01/2008:20418

Ph. Eur. (2011b) Hepatitis A vaccine (inactivataed, adsorbed). In: Pharmacopoeia E (ed) European Pharmacopoeia 7.0

Ph. Eur. (2011c) Influenza vaccine (whole virion, inactivated). In: Pharmacopoeia E (ed) European Pharmacopoeia, 7th ed, pp 795–798

Ph. Eur. (2011d) Poliomyelitis vaccine (inactivated). In: Pharmacopoeia E (ed) European Pharmacopoeia, 7th edn

Ph. Eur. (2011e) Rabies vaccine for human use prepared in cell cultures. In: Pharmacopoeia E (ed) European Pharmacopoeia 7th edn, pp 822–824

Ph. Eur. (2011f) Tick borne encephalitis vaccine (inactivated). In: Pharmacopoeia E (ed) European Pharmacopoeia, 7th edn, pp 834–836

Pittman P, Plotkin SA (2004) Miscellaneous limited-use vaccines. In: Bell BP, Feinstone SM (eds) Vaccines, 4th edn. Saunders Elsevier, Philadelphia

Plotkin SA, Vidor E (2004) Hepatitis A vaccine. In: Bell BP, Feinstone SM (eds) Vaccines, 4th edn. Saunders Elsevier, Philadelphia

Poirier B, Variot P, Delourme P, Maurin J, Morgeaux S (2010) Would an in vitro ELISA test be a suitable alternative potency method to the in vivo immunogenicity assay commonly used in the context of international hepatitis A vaccines batch release? Vaccine 28(7):1796–1802

Polack FP, Auwaerter PG, Lee SH, Nousari HC, Valsamakis A, Leiferman KM, Diwan A, Adams RJ, Griffin DE (1999) Production of atypical measles in rhesus macaques: evidence for disease mediated by immune complex formation and eosinophils in the presence of fusion-inhibiting antibody. Nat Med 5(6):629–634

Polack FP, Hoffman SJ, Crujeiras G, Griffin DE (2003) A role for nonprotective complement-fixing antibodies with low avidity for measles virus in atypical measles. Nat Med 9 (9):1209–1213

Provost PJ, Bishop RP, Gerety RJ, Hilleman MR, McAleer WJ, Scolnick EM, Stevens CE (1986a) New findings in live, attenuated hepatitis A vaccine development. J Med Virol 20(2):165–175

Provost PJ, Hilleman MR (1979) Propagation of human hepatitis A virus in cell culture in vitro. Proc Soc Exp Biol Med 160(2):213–221

Provost PJ, Hughes JV, Miller WJ, Giesa PA, Banker FS, Emini EA (1986b) An inactivated hepatitis A viral vaccine of cell culture origin. J Med Virol 19(1):23–31

Putnak RJ, Coller BA, Voss G, Vaughn DW, Clements D, Peters I, Bignami G, Houng HS, Chen RC, Barvir DA, Seriwatana J, Cayphas S, Garcon N, Gheysen D, Kanesa-Thasan N, McDonell M, Humphreys T, Eckels KH, Prieels JP, Innis BL (2005) An evaluation of dengue type-2 inactivated, recombinant subunit, and live-attenuated vaccine candidates in the rhesus macaque model. Vaccine 23(35):4442–4452

Rappuoli R (1994) Toxin inactivation and antigen stabilization: two different uses of formaldehyde. Vaccine 12(7):579–581

Redman RL, Nader S, Zerboni L, Liu C, Wong RM, Brown BW, Arvin AM (1997) Early reconstitution of immunity and decreased severity of herpes zoster in bone marrow transplant recipients immunized with inactivated varicella vaccine. J Infect Dis 176(3):578–585

Reitman M, Tonik EJ (1971) Immunity to aerosol challenge in guinea pigs immunized with gamma-irradiated Venezuelan equine encephalitis vaccines. Appl Microbiol 21(4):688–692

Reitman M, Tribble HR Jr, Green L (1970) Gamma-irradiated Venezuelan equine encephalitis vaccines. Appl Microbiol 19(5):763–767

Rendi-Wagner P (2008) Advances in vaccination against tick-borne encephalitis. Expert Rev Vaccines 7(5):589–596

Rice WG, Supko JG, Malspeis L, Buckheit RW Jr, Clanton D, Bu M, Graham L, Schaeffer CA, Turpin JA, Domagala J, Gogliotti R, Bader JP, Halliday SM, Coren L, Sowder RC 2nd, Arthur LO, Henderson LE (1995) Inhibitors of HIV nucleocapsid protein zinc fingers as candidates for the treatment of AIDS. Science 270(5239):1194–1197

Roberts A, Lamirande EW, Vogel L, Baras B, Goossens G, Knott I, Chen J, Ward JM, Vassilev V, Subbarao K (2010) Immunogenicity and protective efficacy in mice and hamsters of a beta-propiolactone inactivated whole virus SARS-CoV vaccine. Viral Immunol 23(5):509–519

Roberts JJ, Warwick GP (1963) The reaction of beta-propiolactone with guanosine, deoxyguanylic acid and RNA. Biochem Pharmacol 12:1441–1442

Rooijakkers E, Groen J, Uittenbogarrd J, van Herwijnen J, Osterhaus A (1996a) Development and evaluation of alternative testing methods for the in vivo NIH potency test used for the quality control of inactivated rabies vaccines. Dev Biol Stand 86:137–145

Rooijakkers EJ, Uittenbogaard JP, Groen J, Osterhaus AD (1996b) Rabies vaccine potency control: comparison of ELISA systems for antigenicity testing. J Virol Methods 58 (1–2):111–119

Rossio JL, Esser MT, Suryanarayana K, Schneider DK, Bess JW Jr, Vasquez GM, Wiltrout TA, Chertova E, Grimes MK, Sattentau Q, Arthur LO, Henderson LE, Lifson JD (1998) Inactivation of human immunodeficiency virus type 1 infectivity with preservation of conformational and functional integrity of virion surface proteins. J Virol 72(10):7992–8001

Salk JE (1953) Studies in human subjects on active immunization against poliomyelitis. I. A preliminary report of experiments in progress. J Am Med Assoc 151(13):1081–1098

Salk JE (1955) Vaccination against paralytic poliomyelitis performance and prospects. Am J Public Health Nations Health 45(5 Pt 1):575–596

Salk JE, Francis T Jr (1946) Immunization against influenza. Ann Intern Med 25:443–452

Salk JE, Gori JB (1960) A review of theoretical, experimental, and practical considerations in the use of formaldehyde for the inactivation of poliovirus. Ann NY Acad Sci 83:609–637

Salk JE, Krech U, Youngner JS, Bennett BL, Lewis LJ, Bazeley PL (1954) Formaldehyde treatment and safety testing of experimental poliomyelitis vaccines. Am J Public Health Nations Health 44(5):563–570

Salmon DE, Smith T (1886) On a new method of producing immunity from contagious diseases. Am Vet Rev 10:63–69 (First published)

Schultz-Cherry S, Jones JC (2010) Influenza vaccines: the good, the bad, and the eggs. Adv Virus Res 77:63–84

Schultz P, Rightsel WA, Timm EA, Taylor AR, Mc LI Jr (1957) Partially inactivated poliomyelitis virus: initiation of infection in tissue culture. J Immunol 79(6):497–507

Segal A, Solomon JJ, Mignano J, Dino J (1981) The isolation and characterization of 3-(2-carboxyethyl)cytosine following in vitro reaction of beta-propiolactone with calf thymus DNA. Chem Biol Interact 35(3):349–361

Semple D (1911) The preparation of a safe and efficient antirabic vaccine. Scientific Memoirs by Officers of the Medical and Sanitary Departments of India, vol 44. Superintendent Government Printing, Calcutta. https://archive.org/stream/preparationofsaf00sempuoft#page/n5/mode/2up

Shafique M, Wilschut J, de Haan A (2012) Induction of mucosal and systemic immunity against respiratory syncytial virus by inactivated virus supplemented with TLR9 and NOD2 ligands. Vaccine 30(3):597–606

Skykes G (1965) The theory and mode of action of disinfection. In: Lippincott (ed) Disinfection and sterilization, Philadelphia

Smorodintsev AA, Ilyenko VI (1969) Results of laboratory and epidemiological study of vaccination against tick-borne encephalitis. In: Libíková H (ed) Biology of viruses of the tick-borne encephalitis complex, Smolenice, 11–14 October 1960, pp 332–343

Stauffer F, El-Bacha T, Da Poian AT (2006) Advances in the development of inactivated virus vaccines. Recent Pat Antiinfect Drug Discov 1(3):291–296

Steele KE, Reed DS, Glass PJ, Hart MK, Ludwig GV, Pratt WD (2007) Aplhavirus Encephalitides. In: Dembek ZF (ed) Medical aspects of biological warfare. Office of the Surgeon General, US Army Medical Department Center and School, Borden Institute, Washington DC, pp 241–270

Stokes J, Chenoweth AD, Waltz AD, Gladen RG, Shaw D (1937) Results of immunization by means of active virus of human influenza. J Clin Invest 16(2):237–243

Sugawara K, Nishiyama K, Ishikawa Y, Abe M, Sonoda K, Komatsu K, Horikawa Y, Takeda K, Honda T, Kuzuhara S, Kino Y, Mizokami H, Mizuno K, Oka T, Honda K (2002) Development of Vero cell-derived inactivated Japanese encephalitis vaccine. Biologicals 30(4):303–314

Swanson MC, Rosanoff E, Gurwith M, Deitch M, Schnurrenberger P, Reed CE (1987) IgE and IgG antibodies to beta-propiolactone and human serum albumin associated with urticarial reactions to rabies vaccine. J Infect Dis 155(5):909–913

Termini J (2000) Hydroperoxide-induced DNA damage and mutations. Mutat Res 450 (1–2):107–124

Thaysen-Andersen M, Jorgensen SB, Wilhelmsen ES, Petersen JW, Hojrup P (2007) Investigation of the detoxification mechanism of formaldehyde-treated tetanus toxin. Vaccine 25 (12):2213–2227

Uittenbogaard JP, Zomer B, Hoogerhout P, Metz B (2011) Reactions of beta-propiolactone with nucleobase analogues, nucleosides, and peptides: implications for the inactivation of viruses. J Biol Chem 286(42):36198–36214

USFDA (1998) Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin, Appendix 3. ICH guidance for industry Q5A. United States Food and Drug Administration

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39 (1):44–84

van Steenis G, van Wezel AL, Sekhuis VM (1981) Potency testing of killed polio vaccine in rats. Dev Biol Stand 47:119–128

Verdijk P, Rots NY, Bakker WA (2011) Clinical development of a novel inactivated poliomyelitis vaccine based on attenuated Sabin poliovirus strains. Expert Rev Vaccines 10(5):635–644

Verma R, Khanna P, Chawla S (2012) Influenza vaccine: an effective preventive vaccine for developing countries. Hum Vaccin Immunother 8(5):675–678

Vidor E, Fritzell B, Plotkin S (1996) Clinical development of a new inactivated hepatitis A vaccine. Infection 24(6):447–458

Wang Y, Azevedo M, Saif LJ, Gentsch JR, Glass RI, Jiang B (2010) Inactivated rotavirus vaccine induces protective immunity in gnotobiotic piglets. Vaccine 28(33):5432–5436
Wassilak SGF, Roper MH, Murphy TV, Orenstein WA (2004) Tetanus toxoid. In: Bell BP, Feinstone SM (eds) Vaccines, 4th edn. Saunders Elsevier, Philadelphia
Weller TH, Robbins FC, Enders JF (1949) Cultivation of poliomyelitis virus in cultures of human foreskin and embryonic tissues. Proc Soc Exp Biol Med 72(1):153–155
Westdijk J, Brugmans D, Martin J, van't Oever A, Bakker WA, Levels L, Kersten G (2011) Characterization and standardization of Sabin based inactivated polio vaccine: proposal for a new antigen unit for inactivated polio vaccines. Vaccine 29(18):3390–3397
White (1955) Technical report on Salk poliomyelitis vaccine. U.S. Public Health Service, Washington DC
WHO (1984) WHO expert committee on rabies. World Health Organization technical report series, vol 709
WHO (1995) Requirements for hepatitis A vaccine (inactivated). Requirements for biological substances No. 49. World Health Organization
WHO (1999) Requirements for tick-borne encephalitis vaccine (inactivated). Requirements for biological substances No. 51, WHO technical report series, No. 889, 1999. World Health Organization, Geneva
WHO (2000) Recommended composition of influenza virus vaccines for use in the 2001 influenza season. Wkly Epidemiol Rec 75:61–65. World Health Organization
WHO (2002) Recommendations for the production and control of poliomyelitis vaccine (inactivated). WHO technical report series 910, Annex 2. Geneva
WHO (2005) WHO expert consultation on rabies. World Health Organization technical report series, vol 931. World Health Organization, Geneva
WHO (2006a) Inactivated poliovirus vaccine following oral poliovirus vaccine cessation. Wkly Epidemiol Rec 81(15):137–144
WHO (2006b) Japanese encephalitis vaccines. Wkly Epidemiol Rec 81:331–340 (World Health Organization, Geneva)
WHO (2007a) Recommendations for inactivated rabies vaccine for human use produced in cell substrates and embryonated eggs. WHO technical report series, No 941. World Health Organization, Geneva
WHO (2007b) Recommendations for Japanese encephalitis vaccine (inactivated) for human use. WHO Expert Committee on Biological Standardization. World Health Organization, Geneva
WHO (2009a) Recommended composition of influenza virus vaccines for use in the 2009–2010 influenza season (northern hemisphere winter). Wkly Epidemiol Rec 84:421–431 (World Health Organization, Geneva)
WHO (2009b) Update on improving IPV. The Polio pipeline. World Health Organization, Geneva
WHO (2009c) WHO global action plan to minimize poliovirus facility associated risk after eradication of wild polioviruses and cessation of routine OPV use. World Health Organization, Geneva
WHO (2011) Vaccines against tick-borne encephalitis: WHO position paper. Wkly Epidemiol Rec 86:241–256 (World Health Organization, Geneva)
Wiktor TJ, Aaslestad HG, Kaplan MM (1972) Immunogenicity of rabies virus inactivated by -propiolactone, acetylethyleneimine, and ionizing irradiation. Appl Microbiol 23(5):914–918
Wilde JA, McMillan JA, Serwint J, Butta J, O'Riordan MA, Steinhoff MC (1999) Effectiveness of influenza vaccine in health care professionals: a randomized trial. JAMA 281(10):908–913
Wood JM, Williams MS (1998) History of inactivated influenza vaccines. In: Nicholson KGW, Webster RG, Hay AJ (ed) Textbook of influenza. Blackwell Science, Oxford, pp 324–345
Wright AE, Semple D (1897) Remarks on vaccination against typhoid fever. Br Med J 1 (1883):256–259
Wu X, Smith TG, Rupprecht CE (2011) From brain passage to cell adaptation: the road of human rabies vaccine development. Expert Rev Vaccines 10(11):1597–1608
Zacks MA, Paessler S (2009) Encephalitic alphaviruses. Vet Microbiol 140(3–4):281–286

Zehrung D (2010) Improving the affordability of Inactivated Poliovirus Vaccines (IPV) for use in low- and middle-income countries. An economic analysis of strategies to reduce the cose of routine IPV immunization. PATH, Seattle

Zepp F (2011) Principles of vaccine design-lessons from nature. Vaccine 28(Suppl 3):C14–C24

Zhu FC, Liang ZL, Li XL, Ge HM, Meng FY, Mao QY, Zhang YT, Hu YM, Zhang ZY, Li JX, Gao F, Chen QH, Zhu QY, Chu K, Wu X, Yao X, Guo HJ, Chen XQ, Liu P, Dong YY, Li FX, Shen XL, Wang JZ (2013) Immunogenicity and safety of an enterovirus 71 vaccine in healthy Chinese children and infants: a randomised, double-blind, placebo-controlled phase 2 clinical trial. Lancet 381:1037–1045

Zissis G, Lambert JP, Marbehant P, Marissens D, Lobmann M, Charlier P, Delem A, Zygraich N (1983) Protection studies in colostrum-deprived piglets of a bovine rotavirus vaccine candidate using human rotavirus strains for challenge. J Infect Dis 148(6):1061–1068

Chapter 3
Recombinant Virus-like Particle Protein Vaccines

Robert D. Sitrin, Qinjian Zhao, Clinton S. Potter, Bridget Carragher and Michael W. Washabaugh

3.1 Introduction

Viral diseases offer a major challenge to vaccine development because of the complex nature of virus structures and the large size of the virus particle needed to generate an effective immune response. Viral diseases frequently stimulate both Th2 (antibody-mediated) and Th1 (cell-mediated) immune pathways. Thus, a viral subunit vaccine candidate should stimulate both immune response pathways and might require a novel adjuvant that favors the Th1 cell-mediated pathway. In general, subunit vaccines with smaller molecular weights have failed to elicit a protective immune response for viral targets. Thus, classical approaches to develop antiviral vaccines have required the use of attenuated (or inactivated) live viruses produced in cell culture. Although this approach has been widely successful (measles, mumps, rubella, rotavirus, varicella, polio, and hepatitis A) and is still in practice, not all viruses are amenable to replication in cell culture, particularly at commercial scale.

R.D. Sitrin (✉)
SitrinSolutions, LLC, 237 Emerson Drive, Lafayette Hill, PA 19444, USA
e-mail: Sitrinsolutions@gmail.com

Q. Zhao
State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics,
School of Public Health, Xiamen University, Xiamen 361102, Fujian, China

C.S. Potter · B. Carragher
NanoImaging Services, Inc., 11099 North Torrey Pines Road,
San Diego, CA 92037, USA

C.S. Potter · B. Carragher
Department of Integrative Structural and Computational Biology,
The Scripps Research Institute, La Jolla, CA 92037, USA

M.W. Washabaugh
MedImmune, Inc., Gaithersburg, MD 20878, USA

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6_3

With the advent of modern molecular biology, it has become reasonable to consider the use recombinant DNA approaches to produce modern prophylactic vaccines. One interesting problem presents itself here, as the viruses themselves are fairly large (multimillion Dalton molecular weight), and thus pose a special challenge to cloning such a large entity. In fact, the viruses themselves face the same problem, in that if they needed to package the genome coding for a multimillion Dalton entity, they would not have sufficient room inside their viral capsid structure for such a large genome. They have solved this problem by evolving over thousands of years the ability to produce proteins of modest size (24 kDa and higher), which self-assemble into icosahedral virus particles after viral replication inside a host cell (Harrison 1990). In appropriate expression systems, these proteins retain that ability to self-assemble into virus-like particle (VLP) structures whose surface is immunochemically comparable to that of the actual virus. This has been a major success story in vaccines and has enabled the development and commercialization of vaccines against Hepatitis B (HBV) (Sitrin et al. 1993; Stephenne 1990), Human Papillomavirus (HPV) (Shi et al. 2007), and recently flu (FDA 2013; Cox 2011) and Hepatitis E (HEV) (Zhu et al. 2010).

3.2 Common Issues for Recombinant-Derived VLP Vaccines

3.2.1 Comparison with Other Recombinant Protein Products

In general, production and licensure of recombinant vaccines share many of the issues important in licensure of recombinant therapeutic proteins and monoclonal antibodies. Current licensed VLP vaccines use either yeast or Baculovirus expression systems—both amenable to fermentation or cell culture at relatively large scale (Cox 2012). The expressed proteins self-assemble during production or purification to produce VLP entities, often of 20–80 nm in diameter. The EP monographs (EP 2008, 2010a) describe the expected EU testing and release criteria for HBV and HPV vaccines, which are presumably similar to the corresponding FDA release expectations. As is the common practice for recombinant products, master cell banks and banked viral seed lots are tested and released for freedom from adventitious agents (viral or bacterial as appropriate). At the completion of purification, usually to a >95 % value, products are sterile filtered and formulated onto aluminum salts and other adjuvants. In general, release and characterization testing procedures are analogous to those used in the Biotech industry for recombinant proteins such as purity, and host cell and DNA residuals (EP 2008, 2010a). Final dosage forms are routinely tested for sterility, adjuvant content and potency. (EP 2008, 2010a; WHO 2006; CHMP 2006).

3.2.2 Comprehensive Characterization

Recombinant VLP vaccines, particularly pivotal clinical and process validation lots, are typically subjected to comprehensive testing analogous to typical testing done on therapeutic protein products (purity by SDS-PAGE, primary and secondary structure) as well as further tests to measure tertiary and quaternary structure and properties on aluminum adjuvant. This additional analytical characterization is above and beyond the release testing carried out on commercial lots. It has become increasingly clear that structural and functional methods for analysis of recombinant VLP-based vaccines are beneficial during development and postlicensure (Zhao et al. 2012a; Zhao et al. 2013b). In addition, characterizing in vitro antigenicity, in an epitope-specific manner, is emerging as an excellent surrogate marker for in vivo immunogenicity or vaccine efficacy (Shank-Retzlaff et al. 2005; Zhao et al. 2012b). Figure 3.1 summarizes the analytical procedures used to assess primary, secondary, tertiary, and quaternary protein structure of GARDASIL® and demonstrates the array of testing technology that can be applied to any recombinant VLP-based vaccine (Sitrin 2010).

A major issue for vaccines is the ability to manage process or facility changes at commercial scale without the need for repeated clinical trials, which is often the case for "traditional" vaccines. It is important to establish the link or correlation between critical product attributes and critical process conditions. A well-defined process can result in a well-characterized product. This is particularly important for clinically relevant structural features such as neutralizing and immune-dominant epitopes for a prophylactic vaccine. Thus, the ability to characterize a recombinant VLP vaccine offers an excellent tool box to demonstrate "comparability" to license process or facility changes or upgrades.

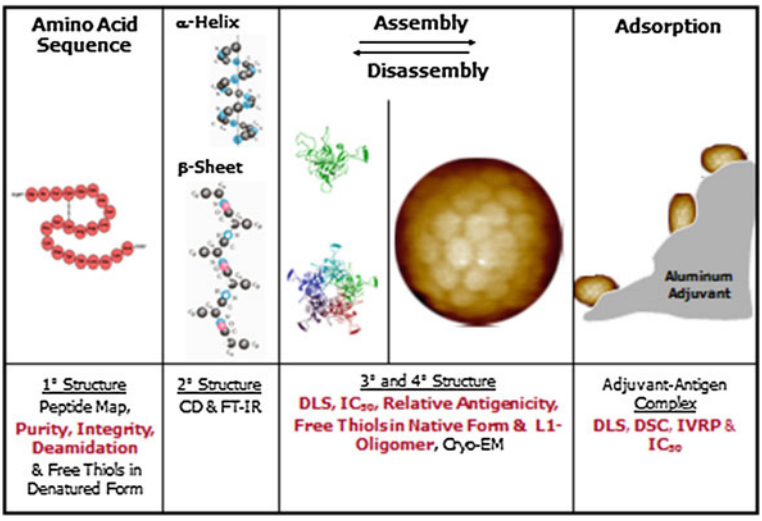


Fig. 3.1 Typical analytical techniques applied to VLP vaccines (Sitrin 2010)

3.2.3 Complexity of Protein Vaccines Versus Small Molecule Drugs

Complexity is the central theme when dealing with the properties of biologics or vaccines. Unlike traditional small molecular weight drugs, where high performance liquid chromatography (HPLC), mass spectrometry (MS), and nuclear magnetic resonance (NMR) can fully define structure and function, high molecular weight biological identities display an array of slightly different variations in structures or conformations which cannot fully define the biological aspects of the structure. They have unique signatures, or fingerprints, made up of naturally occurring variations or a complex mixture. These structural variations include heterogeneity in conformation, partial or full modification of N-terminus, disulfide bond pairing, degree or variations in glycosylation or phosphorylation, and different levels of proteolytic clipping of peptide chains occurring during bioprocessing and/or during storage. The complexity reflected by these arrayed variations in structure or conformation may not translate into differences in function. This is why the terms "comparable" or "equivalent" (vs. "same" or "identical") have been used when describing the properties of different batches of products or products made with slightly different processes or at different scales.

3.2.4 What Is Comparability?

For a given product attribute, there are three facets to consider when it comes to defining comparability criteria: process comparability, analytical comparability, and the link of a change to the clinical outcome ("does a change matter?"). Thus, the premise of a comparability exercise is to gauge the future product attributes, when a scale change or process improvement is implemented, using the database (or the fingerprints defined from the past experience) based on the past clinical and commercial manufacturing batches. A weighted approach needs to be adopted, so more meaningful assays, such as the potency assays or in vitro functional binding assays with close links to clinical outcome, can be treated with higher significance. For HBV and HPV vaccines, mouse potency and mAb based in vitro relative potency assays have been extremely important in defining the product efficacy during product development. Orthogonal methods, preferably gauging the bulk property or the "whole sample" (as opposed to a subset of product) properties, are often employed to better assess the products. The recently published A-Vax case study, which explores the use of Quality by Design (QbD) for a VLP vaccine, discusses weighting in terms of a risk assessment and a control strategy that supports comparability studies (PDA 2012).

Better understanding of the structural basis for the functions of VLPs would lead to improved assays to define the specific "fingerprints" and a link to clinical performance. More quantitative analysis and more complete understanding of the

process should lead to more precise control and management of the chemical or biophysical complexity in a vaccine product. This increased knowledge would in turn help to define the design space during process development and process validation to deliver safe and efficacious products.

3.2.5 Special Technology Applied to VLPs

Potency assay: There are unique attributes and tests applied to VLP vaccines that are not generally used to analyze or test recombinant proteins. One of the most critical attributes of any vaccine is that of the potency determination. Classically, in vivo testing (usually mouse or perhaps rat) has been used for release of vaccine products. The Th1 (cell-mediated) response is becoming increasingly important in evaluating vaccine clinical performance, and the mouse potency assay (in vivo immunogenicity) is usually a serology test that only evaluates the Th2 (total polyclonal antibody-mediated) response in the mouse. Consequently, it has been difficult to establish an in vitro antigenicity—in vivo immunogenicity relationship that is also well-correlated with protective immune response in humans. However, because of their unique icosahedral repeat structures with multiple immunological surface epitopes, properly calibrated enzyme linked immunoassay (ELISA) techniques can be used for in vitro potency evaluation and release. These require extensive discussions with regulatory agencies to assure that they are accepted as clinical surrogates of vaccine efficacy.

Sizing analysis: Another unique testing technology not usually applied to recombinant protein products is nanometer scale size determination, either by dynamic light scattering (DLS) or multiangle laser light scattering (MALLS) often in concert with HPLC size exclusion analysis (HPSEC-MALLS) or field flow fractionation (See references in Table 3.1). Size determination of products, using DLS carried out in a routine manner, provides a facile method to track process consistency over time and provides an early warning of potential shifts in performance manifested by changes in size such as aggregation or oligomerization.

Imaging: Finally, because of their large and symmetrical structure, these viral vaccine products are amenable to imaging using Transmission Electron Microscopy (TEM), both in negative stain as well as in a frozen hydrated state (cryoTEM), and by Atomic Force Microscopy (AFM). TEM methods, in combination with modern computational techniques, can also provide three-dimensional visualizations of these vaccines, their interaction with relevant antibodies, and their appearance when adsorbed to adjuvants. In addition, these orthogonal direct visualization methods aid the interpretation of the data generated by the more indirect light scattering methods.

Table 3.1 Techniques used in the size analysis of VLPs with or without an in-line separation process

Method	VLP/observation	Reference
Methods with in-line separation		
High-performance size exclusion chromatography (HPSEC)		
HPSEC with UV or FL detection	HPV/30–60 nm	Mach et al. (2006)
HPSEC with MALLS	HPV/30–60 nm	Li et al. (2007), MacNair (2005)
Analytical ultracentrifugation (AUC)	HPV/VLP	Mach et al. (2006)
Disc centrifugation	HPV/VLP	Deschuyteneer et al. (2010)
Asymmetric field flow fractionation (FFFF or AF4)	VLP	Pease et al. (2009), Mohr et al. (2013)
Electrospray differential mobility analysis (ES-DMA)	Virus/VLP	Guha et al. (2012)
Methods without separation		
Dynamic light scattering (DLS)		Mohr et al. (2013, Sitrin (2010)
Negative-stain TEM		Shi et al. (2007)
CryoTEM	3D reconstruction	Zhao et al. (2014), Mulder et al. (2012)
Protein tomography		Deschuyteneer et al. (2010)
Atomic force microscopy (AFM)		Zhao et al. (2012a), (2012b), Mulder et al. (2012), Zhao et al. (2006)

3.2.6 VLPs Are "Well-Characterized Vaccines"

Based on its design, this recombinant VLP approach yields an entity that could be considered well characterized, as are the therapeutic protein products that are manufactured in an analogous manner. Thus, unlike the situation for traditional vaccines, a comprehensive characterization data base provides an excellent and well-accepted means to carry out future process and manufacturing facility changes without repeating clinical studies. It should be noted that this database should contain significant analytical characterization information above and beyond release data.

3.3 HBV Vaccines

3.3.1 Serum-Derived Vaccine

Hepatitis B vaccine (HBV) products bridge several generations of technology and demonstrate a steady progression of the application of modern techniques to the analysis and characterization of vaccines. The original HBV vaccine, Heptavax®, was licensed by Merck in 1981 (Hilleman 1993). The vaccine antigen, Hepatitis B

surface antigen (HBsAg), was isolated from human carriers and purified by a lengthy and extensive process to yield a safe and effective vaccine adsorbed to an aluminum adjuvant. Most of the analytical testing was designed to assure safety from potential human viruses likely present in the source serum. In fact, the World Health Organization (WHO) document on requirements for release of the plasma-derived HBV vaccine, focuses mostly on safety tests, with expectations for residual HBV viral DNA, purity by SDS-PAGE (>95 %), and a relative mouse potency requirement (WHO 1988; Grachev and Magrath 1993). The dose was set from clinical trials as 20 μg protein determined by UV, micro-Kjedahl, or Lowry. Potency was measured by an in vivo assay in mice assessed by a total polyclonal antibody response to the antigen. Given the available technology at the time, this represented an appropriate panel of tests to characterize this vaccine product.

The Hepatitis B surface antigen, comprised of an approximately 24 kDa molecular weight protein along with smaller amounts of extended sequences ("Pre S1" and "Pre S2") contains very significant hydrophobic sequences, which cause it to take on lipids from the producing (liver) cells and assemble into a 22 nm VLP. Early characterization of the product used SDS-PAGE, amino acid analysis, Edman degradation, HPLC, TEM (22 nm particles), and analytical ultracentrifugation. (Peterson 1981). Figure 3.2 is a negative-stain TEM micrograph of a clinical lot of Heptavax® (Hilleman 1993). Note the regular spherical particles of the antigen in this figure. Lipids, presumably from the host producing cells (phospholipids, cholesterol, and triglycerides), were critical to structure and immunogenicity as the protein was insoluble in the absence of lipids (Gavilanes et al. 1982; Peterson et al. 1982; Gavilanes et al. 1990).

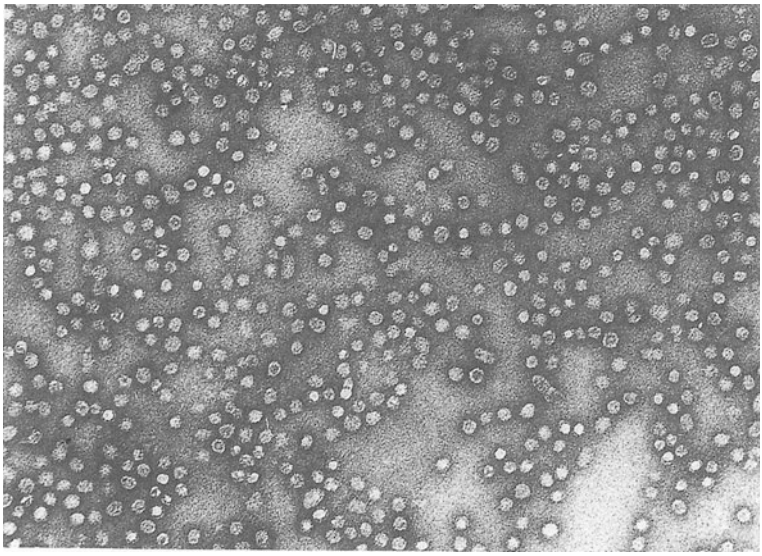


Fig. 3.2 Negative-stain TEM micrograph of Heptavax® (Hilleman 1993). Reprinted with permission

3.3.2 Yeast-Derived Vaccines

Limited supply of source serum and perceived safety issues led to the development of the yeast-derived HBV vaccines developed both by Merck (Recombivax HB®) and GSK (Engerix B®) (Sitrin et al. 1993). These vaccines, expressed in baker's yeast, *Saccharomyces cerevisiae*, were the first application of modern recombinant DNA technology to commercial products. Although the historical safety tests for Heptavax® were no longer required, these new vaccines had to meet all of the newly evolving testing and quality procedures for rDNA products (WHO 1989) with particular focus on purity (SDS-PAGE), acceptable levels of yeast proteins and yeast DNA, as determined by hybridization techniques (<10 ng/dose) (EP 2008; Grachev and Magrath 1993). Although the lipid content changed from human to yeast lipids, the expressed 24 kDa protein was able to assimilate the yeast lipids and make VLPs as effective as the VLP antigen isolated from human carriers. At the time of original licensure, potency was measured by the same technique used for the serum-derived vaccine, a classical ED_{50} determination in mice, using the same specifications (EP 2010b). The product was also tested for sterility, process residuals (formaldehyde or Cesium Chloride), and aluminum (EP 2008) either at the purified bulk or final container stage as appropriate.

3.3.3 Characterization of the Recombinant HBV Vaccines

Both manufacturers applied available technology to characterize their products. Merck reported the use of nonreducing gels to demonstrate the extent of cross-linking of the disulfide containing product into several discrete forms, which were ascribed different levels of antigenicity (Wampler et al. 1985). In GSK publications, the recombinant product was compared to the serum-derived product by EM, amino acid composition, N- and C-terminal data, SDS-PAGE, HPLC, and comparative immunochemistry (Petre et al. 1987; Stephenne 1990). Original data published by GSK (Stephenne 1990) indicated consistent levels of lipid (11–15 mcg per 20 mcg protein), sugars (0.2–0.35 mcg per 20 mcg protein), antigen using a radioimmunoassay called AUSRIA (Abbott_Diagnostics 2012), and protein purity (>98 %) in the bulk product. Further structural studies of the vaccine (Hemling et al. 1988) confirmed 85 % of the sequence by FAB mass spectrometry of proteolytic and CNBr digests. Complete structure confirmation was thwarted by the fact that the protein was 70 % blocked at its N-terminus and contained very hydrophobic sequences making handling of the protein difficult. The lipid-free protein and many of its fragments showed propensity to form water-insoluble aggregates.

3.3.4 Release Strategy and Potency

The doses for the two licensed HBV vaccines are fixed and expressed in terms of protein mass (μg protein as measured by Lowry or the equivalent) (EP 2008; Grachev and Magrath 1993). The protein concentration is measured at a bulk purified stage and this value is used to dilute into the final formulation (adsorption onto aluminum adjuvant). Potency is then measured on the final formulated drug product as a separate measurement from the protein dose. Originally, this potency was determined by an in vivo assay in mice and expressed as an ED_{50} or relative ED_{50} value compared to a standard (Descamps et al. 2011; EP 2010b). An in vitro potency assay based on the Abbott Auszyme Sandwich ELISA kit was later developed to replace the in vivo assay (Descamps et al. 2011). When this kit was discontinued, an in house competitive inhibition ELISA needed to be developed (Descamps et al. 2011). At Merck, an in vitro sandwich ELISA potency test was also developed and validated based on the Abbott Auszyme Sandwich ELISA kit (Schofield 2002). The specifications for Recombivax HB® were based on historical data with Heptavax®. Using extensive concordance data between the two assays (Fig. 3.3), new specifications could be established at the crossover points. This required extensive statistical analysis, given the high variability of the mouse potency assay. Both assays are described in the EP Monograph (EP 2010b).

Both vaccines are formulated on aluminum adjuvant. GSK uses aluminum hydroxide and Merck uses amorphous aluminum hydroxyphosphate sulfate. Final vaccine containers are tested for aluminum, sterility, pyrogens, and potency (EP 2008; Grachev and Magrath 1993).

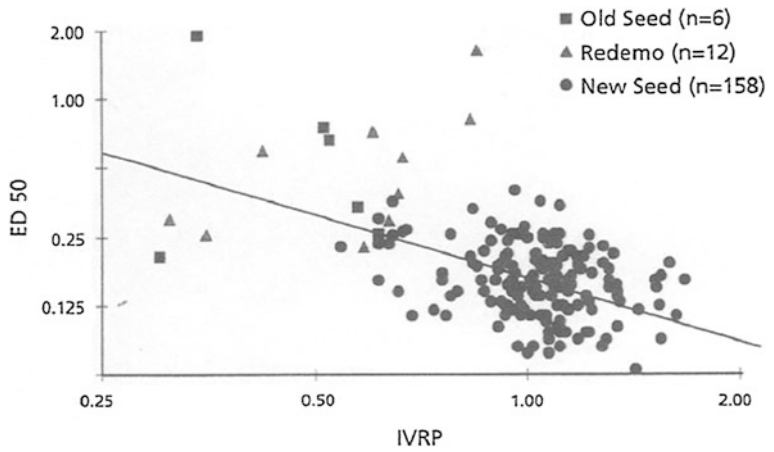


Fig. 3.3 Correlation between mouse potency and in vitro relative potency (IVRP) assays for the recombinant HBV vaccine Recombivax HB®. ED_{50} values are in units of mcg antigen (Schofield 2002). Reprinted with permission

3.3.5 Modern Characterization Methods

Further characterization of the Hepatitis B antigen by more modern techniques has been reported as these have evolved. Using a reversed-phase microisolation procedure and a new matrix 4hcca (4-hydroxy-alpha-cyanocinnamic acid), the sample could be delipidated and the parent molecular ion (25,438 Da) as well as the two major tryptic fragments (13,466 and 11,989 Da could be obtained by MALDI MS (Cohen et al. 2000).

Insights into the maturation of the HBsAg particle and formation during the manufacturing process were observed using atomic force microscopy (AFM) and nonreduced SDS gels (Zhao et al. 2006). Progressive formation of the appropriate disulfide bonds after thiocyanate treatment could be observed as well as changes in particle flexibility. These are demonstrated in the AFM images in Fig. 3.4 where Samples A and B are the antigen before and after thiocyanate treatment. The AFM technique consists of a cantilever with a sharp tip (probe) at its end that is used to scan the specimen surface. When the tip is brought into proximity of a sample surface, forces between the tip and the sample lead to a deflection of the cantilever. These images were obtained in aqueous environments to minimize the deformation of the lipoprotein particles. The lower figures show the expanded region from the

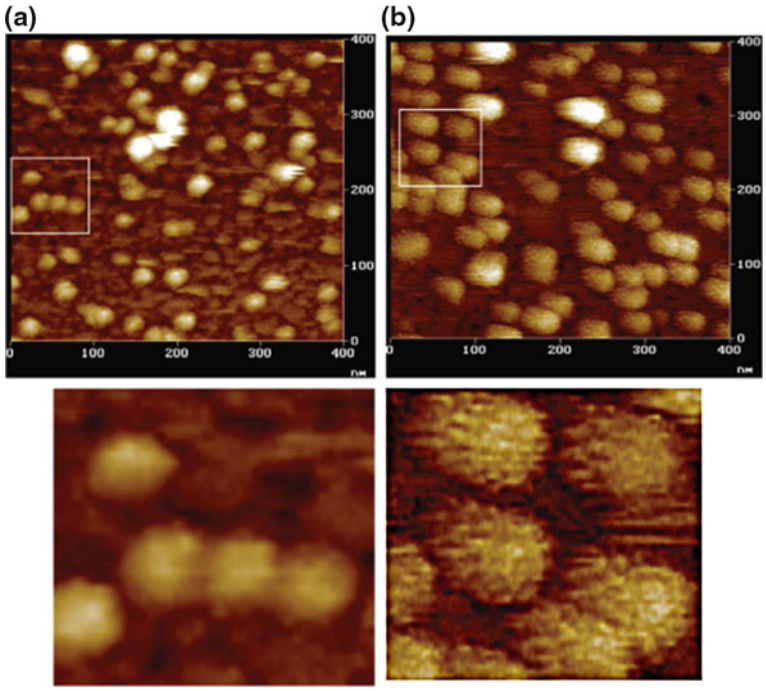


Fig. 3.4 Atomic force microscopy (AFM) images of HBsAg before (**a**) and after (**b**) thiocyanate treatment (Zhao et al. 2006). Reprinted with permission

specified portion of the top figures. Note that the protruded surface features of the HBsAg particles are much better defined after KSCN-induced oxidative maturation with more interchain cross-linking via disulfide bonds (Wampler et al. 1985). Size characterization and monitoring of aggregation during processing steps could be monitored by CD and HPSEC-MALLS (Li et al. 2007).

Further studies on the maturation of the HBsAg particle during manufacture were carried out using immunochemical techniques by surface plasmon resonance (SPR) with monoclonal antibodies known to bind clinically relevant sites on the VLP surface (Zhao et al. 2011a). These tools could also be used to optimize the vaccine antigenicity and immunogenicity using heat treatment or treatment with redox mixtures and to enable rapid process monitoring of manufacturing process intermediates (Zhao et al. 2011b). These modern techniques were summarized as a "Toolbox" for structural and functional characterization of vaccines that includes AFM and CryoTEM studies (Mulder et al. 2012) and so would they fulfill the purpose to demonstrate comparability as mentioned earlier.

3.3.6 CryoTEM Studies

The advantage of CryoTEM is that it provides a very direct means of observing individual particles in a hydrated sample, simultaneously providing information on size, shape, morphology, degree of preservation, and aggregation state. It can also be used to determine the three-dimensional structure of the particles and their interaction with antibodies, and reveal the mode of interaction between the particles and their adjuvants (Fox 2012). In this method, a small drop (3 μL of the sample is placed onto an EM grid covered by a holey carbon support film. The bulk of the sample is removed to leave a thin film, which is then rapidly plunged into a cryogen (e.g., liquid ethane) capable of vitrifying the sample (i.e., freezing it without creating ice crystals). The sample preserved in this vitrified medium is transferred into the electron microscope using a cryogenic stage that maintains the sample temperature below −170 °C. In order to limit damage to the specimen by the electron beam, images are acquired using a very low dose of electrons resulting in images with a low signal-to-noise ratio and limited contrast. The particles are preserved in their natural hydrated state, and thus images provide an accurate description of the morphology of the structures and can be used to extract quantitative metrics such as size distributions and particle fraction counts.

One of the unique advantages of the CryoTEM method is that a three-dimensional map of the structure can be reconstructed by combining particles of the same morphology and conformation but in different relative orientations. The method is generally referred to as "single particle analysis" (described in a recent review (Orlova and Saibil 2011)) and relies on acquiring a large number of images of the randomly oriented particles, which are two-dimensional projections of the three-dimensional object. The individual particles are boxed out of the images and iterative computational methods are used to determine the relative orientation of each

of the two-dimensional projections, which are then backprojected to provide the three-dimensional object. An alternative method, known as electron tomography can also provide three-dimensional reconstructions, although usually at a lower resolution. Analogous to common medical imaging technologies (such as computed tomography), electron tomography acquires a series of images as the sample is rotated in known angular increments about an axis perpendicular to the electron beam. Computational methods are then used to align the images and backproject them to reconstruct the three-dimensional object (Milne et al. 2013).

Yeast-derived HBsAg particles observed by CryoTEM show fairly regular, mostly spherical particles with slightly amorphous boundaries (Fig. 3.5) (Mulder et al. 2012). Further analysis using two-dimensional classification reveals that the VLP surface shows slight protrusions arranged in a regular pattern (Fig. 3.5 inset). Particle-size distribution analysis provides a maximum particle Feret diameter ranging from 18.5 to 22.7 nm (266 particles) and a circularity measure of 0.89 ± 0.03, indicating that the VLP likely deviates from a perfect sphere. Three-dimensional reconstructions show that particles are structured as an empty spherical shell with 24 "knuckle"-like protrusions projecting from a smooth surface and separated by regions of lesser density (Fig. 3.6a, b) (Mulder et al. 2012). The body of each protrusion (presumed to be S-protein) is partially submerged in what is presumed to be lipid monolayer. A region of density corresponding to $\sim$18 kDa of protein, protruded into the extracellular space (Fig. 3.6c, d); notably this region of density would be able to accommodate a tetramer of a stretch of amino acid residues (aa 105–156) in the antigenic loop most implicated in infectivity and antigenicity (Le Duff et al. 2009; Salisse and Sureau 2009). Together with previously published results, the analysis of the three-dimensional cryoTEM map

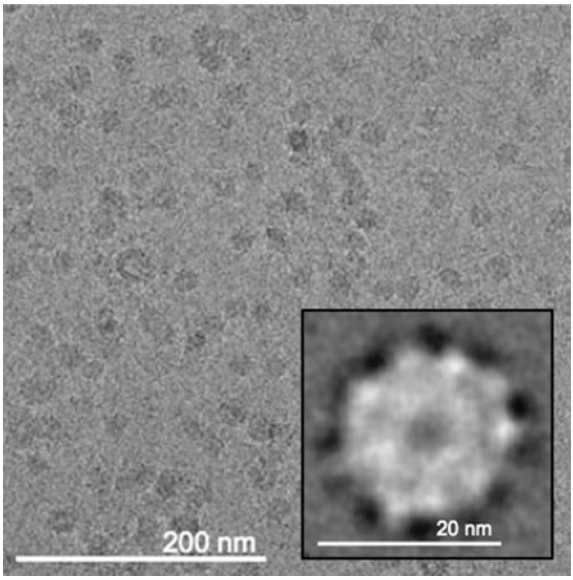


Fig. 3.5 CryoTEM image of HBsAg VLPs. Inset is a two-dimensional class average (Mulder et al. 2012). Reprinted with permission

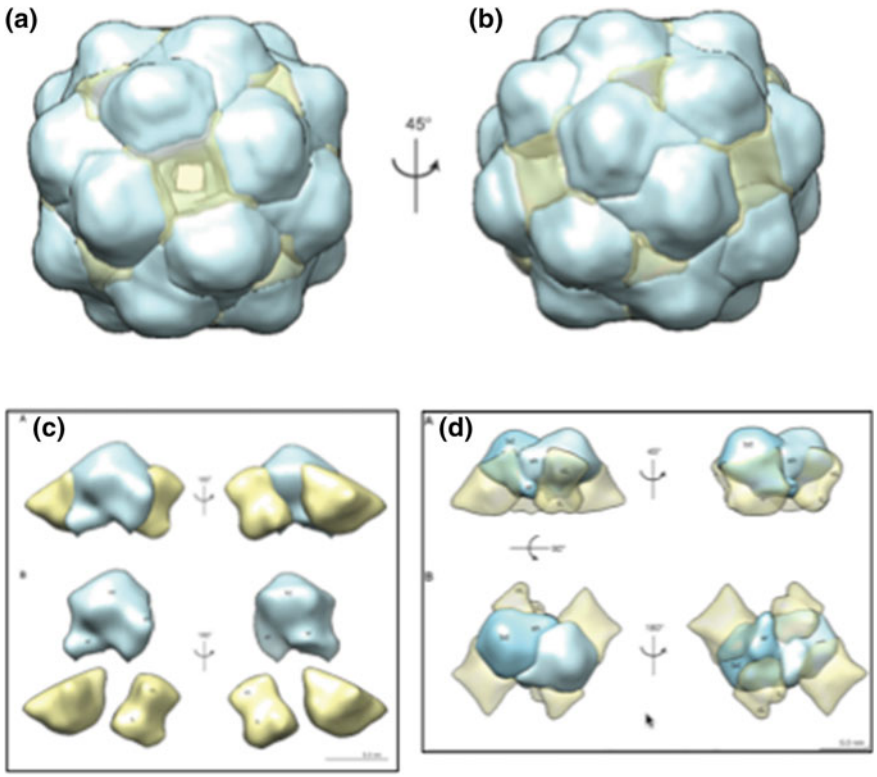


Fig. 3.6 **a/b** CryoTEM map of HBsAg. **c** Structural features of protein containing protrusion and surrounding lipid layer. **d** Arrangement of protein and lipid in the HBsAg particle (Mulder et al. 2012). Reprinted with permission

provides a structural model for the HBsAg vaccine particle that is consistent with previous results regarding the antigenic features, lipid–protein arrangement, and overall particle architecture (Mangold et al. 1995; Stirk et al. 1992; Le Duff et al. 2009; Salisse and Sureau 2009; Gilbert et al. 2005; Greiner et al. 2010; Short et al. 2009).

3.3.7 Closing Comments of HBV

The three decades of HBV vaccine history offer an excellent case study for progressive improvements in technology applied to the same product. Continuous improvement in analytical methods during life cycle management is essential as technologies in the field evolve. The use of these newer analytical techniques provides scientists with new tools to improve processes and production steps and providing additional assurance that a reliable supply of vaccines is maintained.

3.4 HPV Vaccines

3.4.1 History

After over 13 years of development, the U.S. Food and Drug Administration (FDA) approved in 2006 the first preventive HPV vaccine, GARDASIL®, marketed by Merck & Co. (Shi et al. 2007). Glaxo SmithKline gained approval of its HPV vaccine, known as CERVARIX®, in 2007 in Australia, and the European Union, followed by approval in the US in 2009 (Deschuyteneer et al. 2010). HPV vaccines are now licensed in over 100 countries. Since their introduction, the yeast-derived recombinant quadrivalent HPV L1 (Types 6, 11, 16, and 18) vaccine GARDASIL® and the Baculovirus-derived bivalent vaccine (Types 16 and 18) CERVARIX® have played an important role in lowering HPV infection rates in teenage girls (Markowitz et al. 2013) to ultimately reduce incidence of cervical cancer (Dunne and Datta 2008). It is notable that CryoTEM images were used to visualize what the vaccines look like to the general public as evidenced by Fig. 3.7 which was included in a US News and World Report cover story article on the breakthrough launch of anticancer HPV vaccines (Fischman 2006).

For both products, the development strategy was to clone the L1 protein (~55 kDa) specific for each serotype in a recombinant expression system, either full length L1s in yeast (Merck) or as C-terminal truncated proteins in a Baculovirus expression system (GSK). For all of the HPV serotypes, the L1 protein contains in its structure the ability to assemble to capsomers of five monomers (MW ~ 300 kDa),

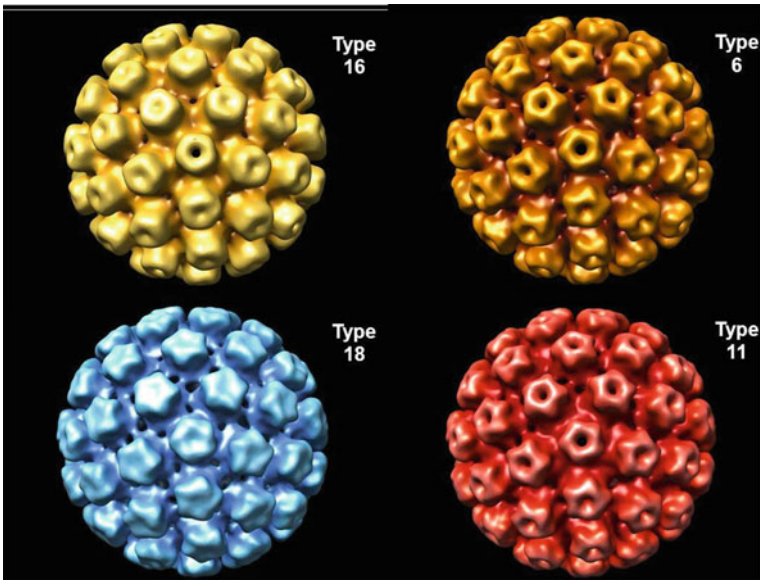


Fig. 3.7 CryoTEM reconstructions of GARDASIL® (Fischman 2006). Reprinted with permission

which themselves assemble into icosahedral structures. The final vaccines contain these VLPs of about 60 nm in size, which mimic the natural viruses in antigenicity and immunogenicity. For both products, the vaccines are purified either as capsomers or VLPs by a variety of chromatographic techniques, with a disassembly/reassembly (D/R) step added at the end for the Merck vaccine process for some types to further enhance the particle morphology, homogeneity, and stability. Both products are formulated on aluminum adjuvant with the GSK product also containing an additional novel adjuvant (ASO4) to enhance its immunogenicity (Deschuyteneer et al. 2010).

3.4.2 Biochemical Analysis

Using established techniques (Fig. 3.1) for recombinant protein characterization, the primary structure of the constituent L1 proteins could be confirmed for both vaccines. Reduced SDS-PAGE was used to confirm the molecular weights and purities (Deschuyteneer et al. 2010). Purified L1 VLPs were analyzed by MALDI-TOF MS on peptide fragments, ISOQUANT® (deamidation), free thiol groups, circular dichroism (CD), and Fourier Transform Infrared (FT-IR) spectroscopy (WHO 2006; CHMP 2006). No evidence has been found of N-linked glycosylation since the observed mass values in SDS-PAGE and in MALDI-TOF MS closely matched the theoretical values (Cohen et al. 1999). GSK published data on peptide mapping of the L1 proteins by tandem LC-MS/MS, N- and C-terminal determinations, Differential Scanning Calorimetry (DSC), TEM (including samples on aluminum adjuvant), CryoTEM, amino acid analysis, disc centrifugation, and relative antigenicity (Deschuyteneer et al. 2010).

3.4.3 Sizing Analysis of VLP-Based Vaccines

Particle size is a key attribute unique to VLP vaccines. A variety of techniques have been applied to determine size and size distribution as shown in Table 3.1. These techniques are particularly helpful to monitor consistency of full-scale manufacturing lots either by DLS for GARDASIL® as shown in Fig. 3.8 (Sitrin 2010) or disc centrifugation for CERVARIX®, as shown in Fig. 3.9 (Deschuyteneer et al. 2010).

3.4.4 Analytical Tools Applied to the Disassembly–Reassembly Process Products

In the GARDASIL® manufacturing process for some of the types, the purified VLP is subjected to a disassembly–reassembly (D/R) step in order to create a more stable and properly assembled particle (Mach et al. 2006). Since this step imparts significant

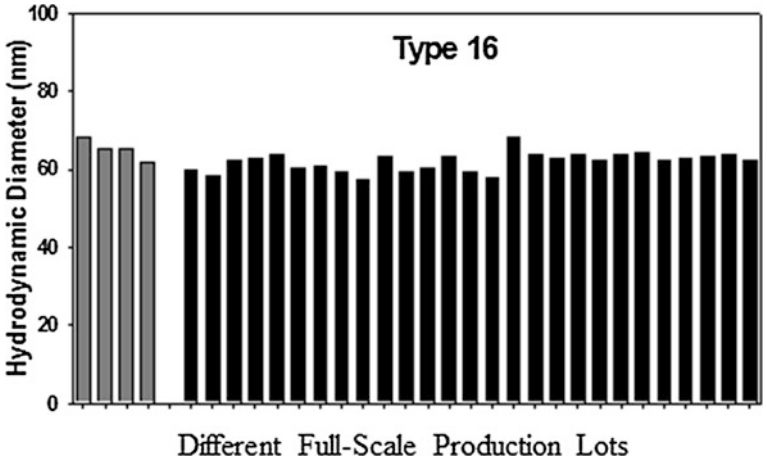


Fig. 3.8 Use of dynamic light scattering (DLS) to monitor consistency of manufacture of VLP vaccine (Sitrin 2010)

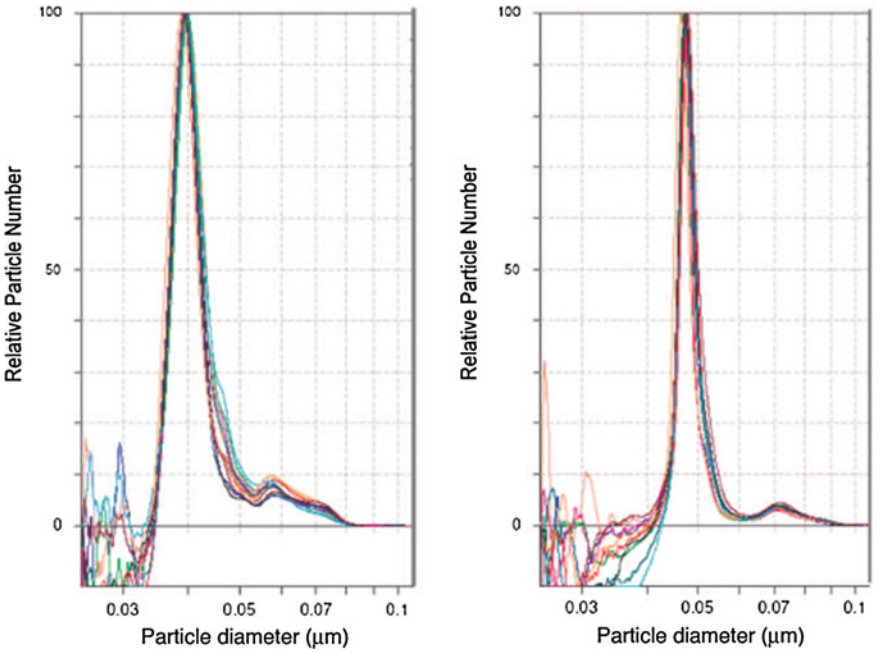


Fig. 3.9 Use of disc centrifugation to monitor consistency of manufacturing of CERVARIX® types 16 (*top*) and 18 (*bottom*) involving ten batches (one color/batch) (Deschuyteneer et al. 2010). Reprinted with permission

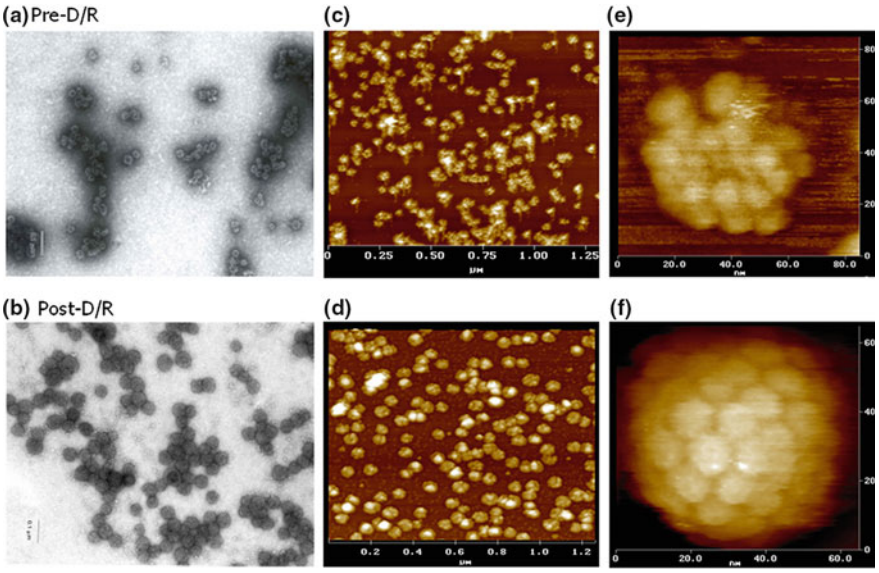


Fig. 3.10 Changes in morphology of VLPs before and after disassembly/reassembly (D/R) as measured by negative-stain TEM (panels **a** & **b**), and atomic force microscopy (AFM) (panels **c–f**) (Zhao et al. 2012b). Reprinted with permission

improvements in stability and potency, it is important to determine which characterization tools can discern between VLPs before and after D/R as they would have verified utility for meaningful parameters. A variety of analytical techniques were applied to observe the VLPs before and after reassembly. For example, Fig. 3.10 (Zhao et al. 2012a) shows the differences by negative-stain TEM as well as by AFM. The changes from irregular to well-defined icosahedral structures are particularly evident in the AFM images. These techniques are especially useful to characterize pivotal samples during process development and manufacturing process demonstrations.

The improved stability of the VLPs after D/R was evident on differential scanning calorimetry (DSC) analysis, which measures thermal transitions as a sample is warmed. A significant upward shift in the lower transition temperature from 53° to 57° as well as a significant change in the relative observed peak heights could be observed with four different batches before and after D/R (see Fig. 3.11) (Zhao et al. 2012a). (Shank-Retzlaff et al. 2006). The results of a cloud point study also supported the decreased propensity for post-D/R VLPs to aggregate, as compared to pre-D/R VLPs, during thermal treatment (Mach et al. 2006).

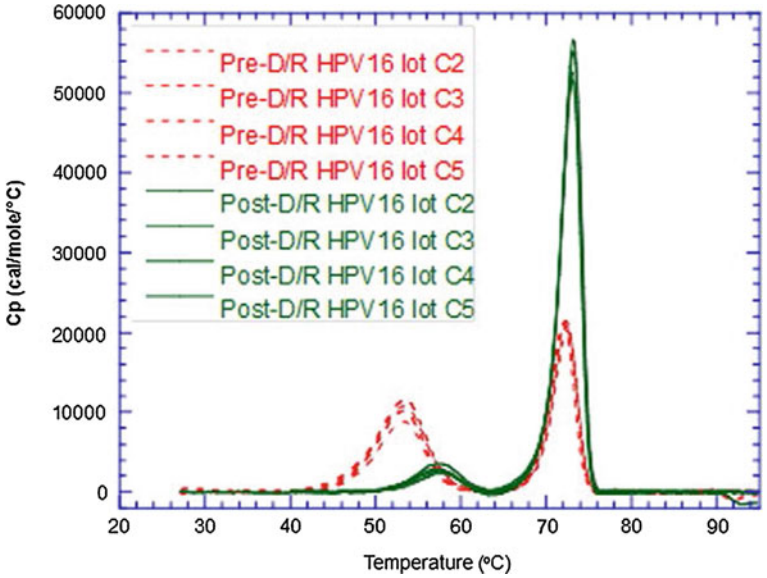


Fig. 3.11 Differential scanning calorimetry (DSC) thermograms of four HPV-16 VLP lots of pre-D/R (traces in *dash lines—red*) and post-D/R treatment (traces in *solid lines—green*). D/R refers to disassembly/reassembly (Zhao et al. 2012a). Reprinted with permission

3.4.5 Immunochemical Characterization and Antigen Quantitation

For GARDASIL®, a panel of highly specific monoclonal antibodies (mAbs) against L1 protein were used to characterize the purified recombinant VLPs in a set of various assays, namely, epitope mapping using label-free SPR technology for the relative footprint size/degree of overlap of different mAbs, inhibitory concentration at 50 % response (IC_{50}) by competitive ELISA (Zhao et al. 2012b) and affinity of mAbs to the different epitopes (solution K_D determination) (Towne et al. 2013). The GSK group demonstrated by ELISA that the same protective epitopes were present in their truncated L1 VLPs as in the full-length L1 pseudovirions (Fig. 3.12) (Deschuyteneer et al. 2010) and demonstrated binding of protective antibodies to their truncated VLPs using SPR.

3.4.6 Immunochemical Analysis of D/R Products

Epitope mapping was performed on the different HPV types prior to and after D/R using an SPR technique with a panel of mAbs that recognizes conformational or linear epitopes. Along with significant improvements in morphology and stability

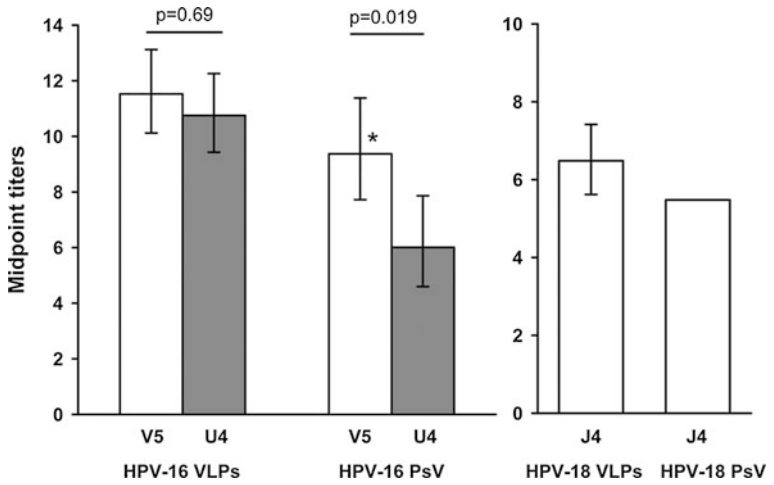


Fig. 3.12 Binding capacity of monoclonal antibodies to truncated L1-derived HPV-16/18 VLPs and HPV-16/18 pseudovirions (PsV), as determined by ELISA (Deschuyteneer et al. 2010). Reprinted with permission

profiles, post-D/R VLPs showed markedly higher antigenicity (~2 to 3.5 fold) to conformational and neutralizing mAbs, whereas the binding to mAbs recognizing linear epitopes was greatly reduced and for one mAb below the detection threshold (Fig. 3.13a) (Zhao et al. 2012b; Towne et al. 2013). A much more focused epitope map was observed for the postreassembly VLPs indicating the decreased heterogeneity in different VLP forms (VLP to VLP) and within VLP conformational heterogeneities (Fig. 3.13b).

3.4.7 Potency Assay

For any new vaccine product, critical attention is required to developing a suitable potency assay and assuring it correlates with clinical performance. This can be particularly challenging for a new vaccine product where a clinical correlate does not yet exist.

3.4.7.1 Dose Versus Potency

As with the case of the Hepatitis B vaccines described earlier, both commercial HPV vaccines express their doses in terms of protein mass (μg protein as measured by Lowry or the equivalent). The protein concentration is measured at a bulk purified stage and this value is used to dilute into the final formulation (adsorption onto aluminum adjuvant). Relative potency is then measured on the final formulated drug

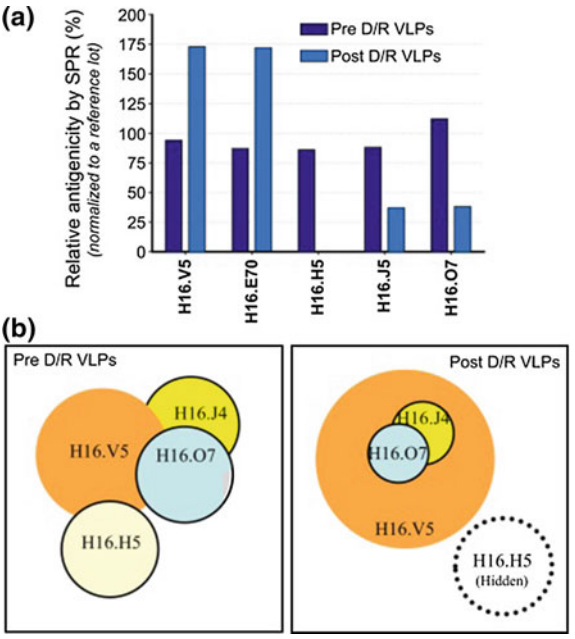


Fig. 3.13 **a** Relative immunochemical changes of HPV-16 before and after disassembly/reassembly (D/R). **b** Relative footprint before and after D/R (Zhao et al. 2012b). Reprinted with permission

product as a separate measurement from the protein dose (EP 2010a) by comparison to a reference (clinical) lot. Originally, this potency was determined by an in vivo assay in mice and expressed as an ED_{50} or relative ED_{50} value compared to a standard. For GARDASIL®, a unique pair of mAbs for each type was selected to develop a type-specific sandwich ELISA assay for measuring the specific antigenicity of a given VLP preparation in aqueous solution. This ELISA was then reformatted to become the in vitro Relative Potency (IVRP) assay on the (aluminum adjuvant-adsorbed) final vaccine product after dissolution treatment and comparison to a reference standard lot (Shank-Retzlaff et al. 2005). Because four different pairs of mAbs were used, each antigen could be tested for release and stability in the presence of the others in the final container. For CERVARIX®, the original in vivo assay used during program development was also replaced by an in vitro ELISA assay which was very effective for demonstrating long-term stability of the vaccine (Le Tallec et al. 2009).

3.4.7.2 Analyzing Adjuvanted Vaccines

Analyzing antigens on adjuvanted vaccines is not as straightforward as analyzing antigens in solution. The quadrivalent vaccine, GARDASIL®, uses amorphous aluminum hydroxyphosphate sulfate adjuvant, while the bivalent CERVARIX® vaccine uses AS04 (500 μg aluminum hydroxide 50 μg 3-O-deacyl-4'-monophosphoryl lipid A) (Deschuyteneer et al. 2010). Adsorbed VLPs have been analyzed by

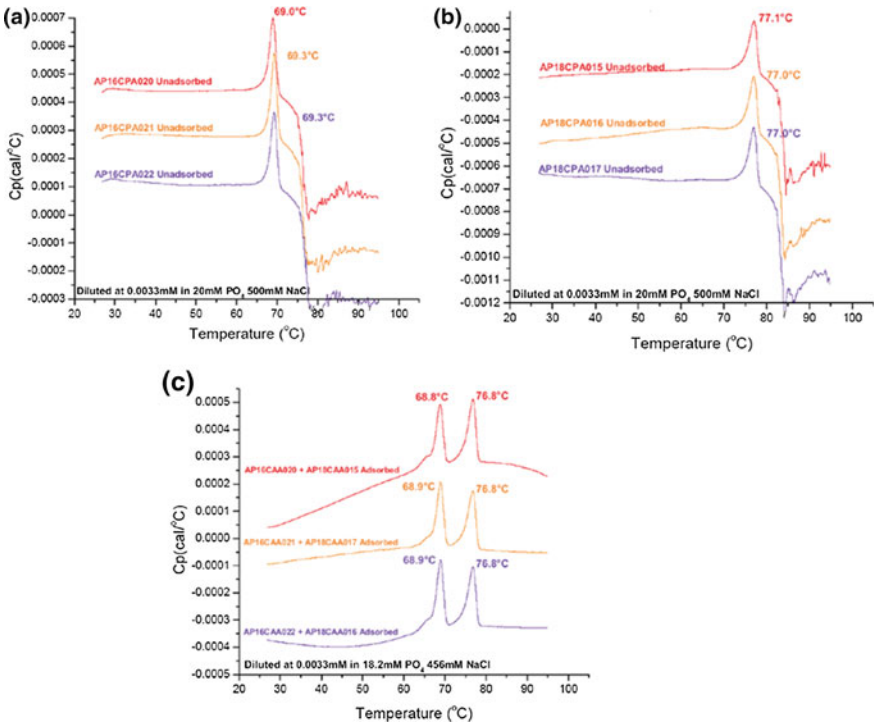


Fig. 3.14 Differential scanning calorimetry (DSC) thermograms of VLPs in solution (**a** and **b**) and of VLPs adsorbed on aluminum hydroxide (**c**). **a** DSC profiles of three different batches of HPV-16 L1 VLPs. **b** DSC profiles of three different batches of HPV-18 L1 VLPs. **c** DSC profiles of three different batches of mixed aluminum-adsorbed HPV-16 and HPV-18 L1 VLPs (Deschuyteneer et al. 2010). Reprinted with permission

mouse potency, IVRP as well as DSC (Deschuyteneer et al. 2010; Shank-Retzlaff et al. 2005). The GSK team used DSC analysis to demonstrate consistency of manufacturing and stability of VLPs when adsorbed to aluminum hydroxide (Fig. 3.14) (Deschuyteneer et al. 2010; Le Tallec et al. 2009). It was concluded that adsorption to the aluminum adjuvant did not result in significant changes in VLPs structure as evidenced by similar morphology and antigenicity pre- and post-adjuvant adsorption. For further discussion on this, see later section for TEM and CryoTEM of VLPs on aluminum adjuvants without pretreatment.

3.4.7.3 Correlation Between in Vivo and in Vitro Assays

For GARDASIL®, the mouse potency (ED_{50}) assay was performed on BALB/c mice in which anti-HPV antibodies were measured using an ELISA-based assay 4 weeks postimmunization. This in vivo potency assay was used to establish the

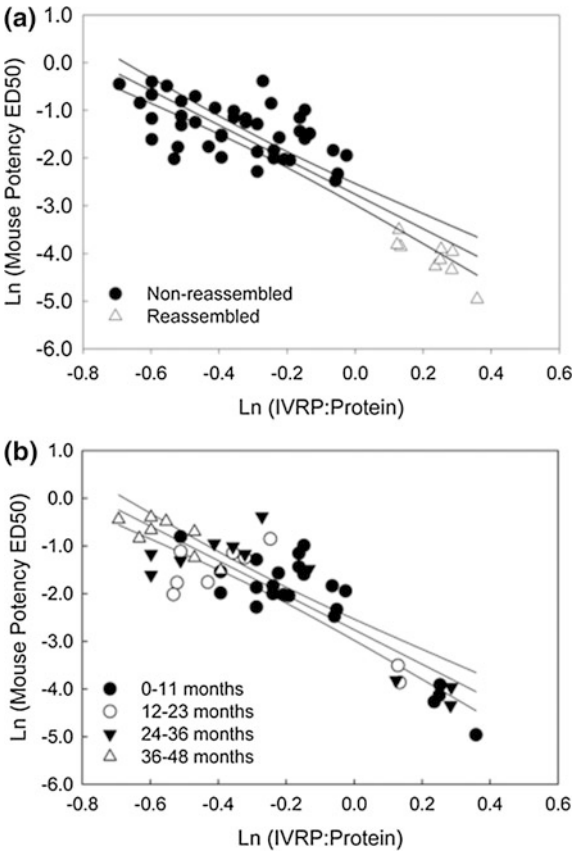


Fig. 3.15 Comparison of in vitro (IVRP) and in vivo (mouse potency) assays for HPV-16 in GARDASIL®. ED_{50} values are in units of mcg antigen **a** plotted by sample type (non-reassembled vs reassembled) and **b** by sample age (months) (Shank-Retzlaff et al. 2005). Reprinted with permission

immunogenicity of formulated products in mice. Both IVRP and in vivo mouse potency assay (ED_{50}) were used to release clinical lots and to characterize the product stability. By the time of licensure, the correlation between the in vivo and in vitro assays had been demonstrated and the IVRP assay (see Fig. 3.15) was approved as the release potency assay (Shank-Retzlaff et al. 2005).

3.4.7.4 Setting Specifications

With the correlation between mouse potency and the IVRP assays established, a novel "quality-by-design" approach was used to set specifications for GARDASIL®. In this study, a statistical evaluation of variability of the ELISA assay and the formulation/filling process along with stability variability was used to propose specifications for the ELISA (see Fig. 3.16) that were confirmed with a specially designed dose-ranging clinical trial (Capen et al. 2007).

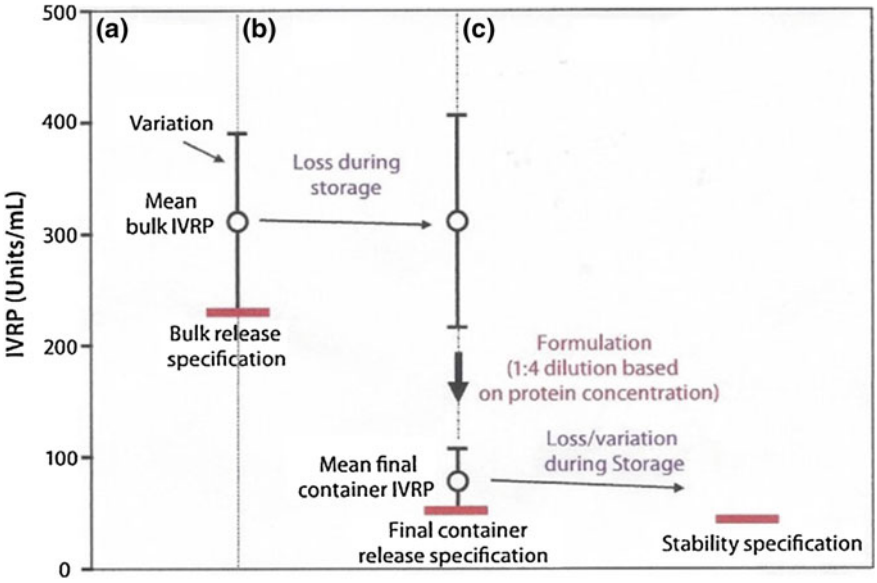


Fig. 3.16 Model used for setting specifications for GARDASIL®. IVRP refers to the in vitro relative potency assay used to measure vaccine potency (Capen et al. 2007). Reprinted with permission

3.4.8 CryoTEM Studies

3.4.8.1 CryoTEM Imaging and Analysis

CryoTEM (see earlier description in Sect. 3.3.6 above) was used to image each of the four GARDASIL® HPV types as shown in Fig. 3.17 (Zhao et al. 2014). The majority of the VLPs appear to be fully assembled, are observed to have a range of sizes, are predominantly spherical or ellipsoidal in shape, with no evidence of filaments or other large aggregates. These observations are similar to those reported using negative-staining EM for the C-terminal truncated VLPs in CERVARIX® (Deschuyteneer et al. 2010).

A set of particles of similar diameter (54 ± 3 nm) were selected from images of VLPs of type 11 and type 16 and single particle analysis methods were used to reconstruct three-dimensional maps of these serotypes as shown in Fig. 3.18a, c (Zhao et al. 2014).

3.4.8.2 VLPs Interacting with Fabs

CryoTEM and single-particle 3D reconstruction was also applied to VLPs interacting with monoclonal antibodies (mAbs), which were known to be protective and

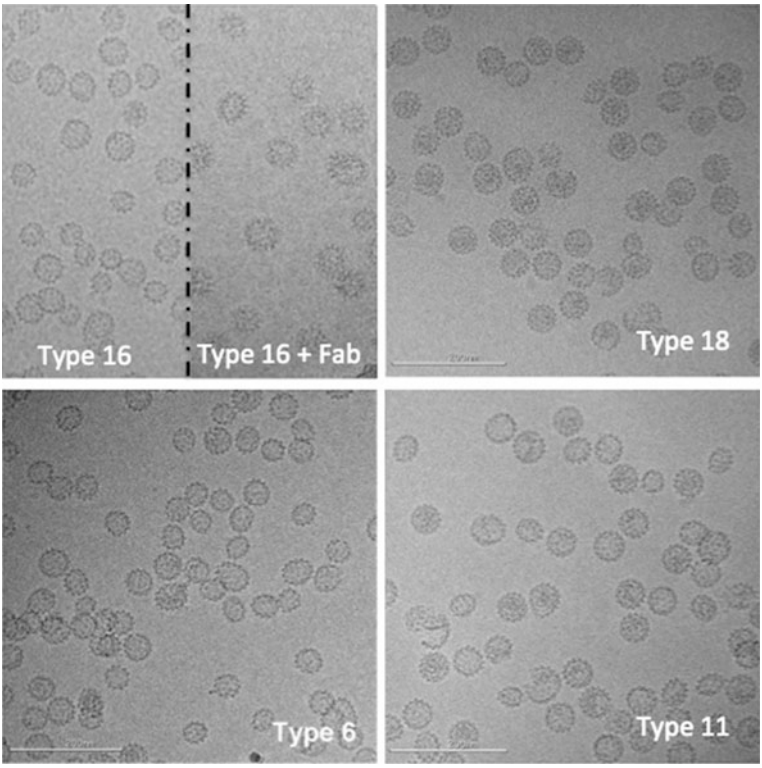


Fig. 3.17 CryoTEM images of VLPs in GARDASIL® (Zhao et al. 2014). Reprinted with permission

which had been used in the potency assay. To avoid cross particle binding, the mAbs were digested and then purified into their respective Fabs. After three-dimensional reconstruction and difference mapping, VLPs can be observed directly interacting with antibody fragments (see Fig. 3.17 inset). Three-dimensional volumes of decorated particles for two different serotypes were reconstructed using methods as described above in Sect. 3.3.6. The antigenic site of interaction is quite different for the two serotypes and their two individual antibodies; for Type 11, the antigenic site is located at the center of the capsomer (Fig. 3.18b), whereas for the Type 16 serotype, the antigenic site for this antibody is located off to the side at the outer edge of the capsomers (Fig. 3.18d) (Zhao et al. 2014).

3.4.8.3 VLPs Interacting with Alum Adjuvant

Images of the VLPs adsorbed to aluminum adjuvant were obtained using CryoTEM (Fig. 3.19) for GARDASIL® and using negative-staining TEM (Fig. 3.20) for CERVARIX®. In both cases, intact VLPs can be observed attached to the adjuvant

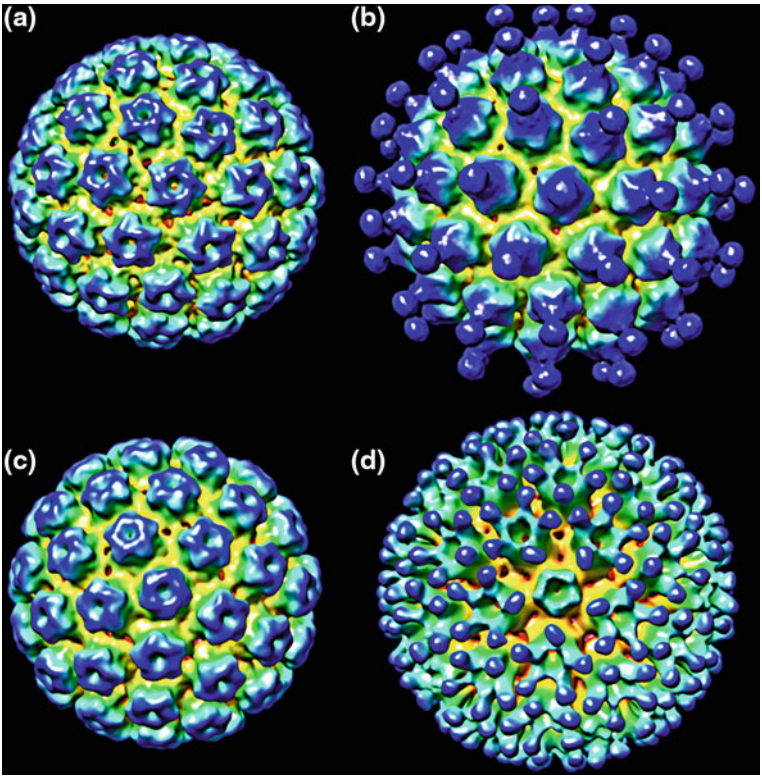


Fig. 3.18 Three-dimensional reconstructions of **a** HPV-11; **b** HPV-11 decorated with antibody fragment H11.B2; **c** HPV-16; and **d** HPV-16 decorated with antibody fragment H16.V5 (Zhao et al. 2014). Reprinted with permission

particles. For GARDASIL®, tomographic methods were used to obtain three-dimensional reconstructions of the structures to confirm that the morphology of the individual is unchanged on binding to the adjuvant (Murphy and Jensen 2007).

3.4.9 Comparability Testing for Life Cycle Management

Extensive characterization provides a database to confirm whether future process changes yield a comparable product. It is critical to determine which tests confirm important similarities or differences. For the GARDASIL® VLP, notable changes could be observed before and after disassembly/reassembly for the TEM, Cryo-TEM, AFM, DLS, HPSEC, DSC, and stability profiles as well as the relative response of various surface epitopes. Since improved antigenicity, stability, mouse potency, and clinical performance were also improved after the D/R step, the utility of these assays for future comparability studies was verified.

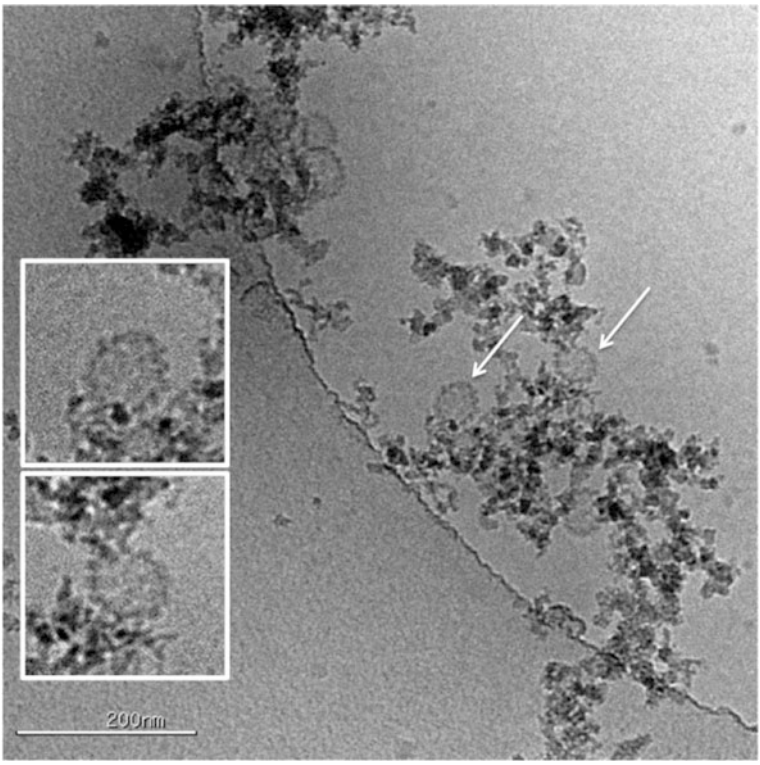


Fig. 3.19 HPV VLPs can be observed directly interacting with the alum adjuvant. The VLPs indicated by the *arrows* are shown at larger scale in the insets indicating that the VLPs are intact and retain their morphology and *spherical shape* upon absorption onto the adjuvant (Zhao et al. 2014). Reprinted with permission

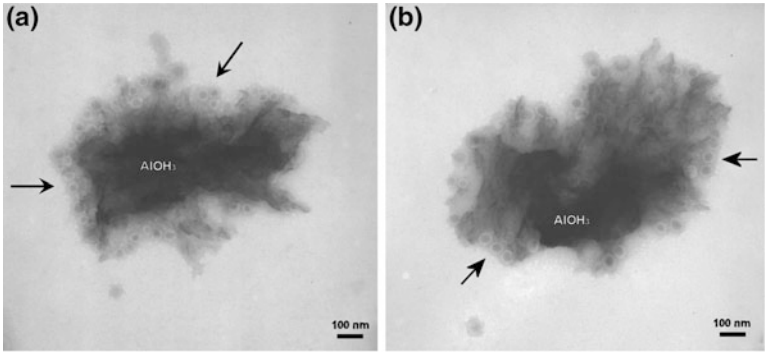


Fig. 3.20 Negative-stain TEM pictures of CERVARIX®, showing that HPV-16 (**a**) and -18 (**b**) L1 VLPs (*arrows*) retain their structure when adsorbed onto aluminum hydroxide (Deschuyteneer et al. 2010). Reprinted with permission

3.5 HEV VLP Vaccine

Recently, a recombinant VLP-based vaccine against Hepatitis E virus infection, Hecolin®, was licensed and launched in China (Zhao et al. 2013a) after demonstration of vaccine safety and efficacy with over 115,000 volunteers enrolled in a large-scale Phase III trial (Zhu et al. 2010). The key antigenic determinants reside on the viral capsid comprised of a single protein encoded by ORF2. Conformation epitopes are essential to mimic the virion surface for desired antigenicity and immunogenicity of the vaccine antigen as evidenced by the antigen-binding activity to a panel of neutralizing mAbs (Zhang et al. 2005; Li et al. 2009). Analytical centrifugation (AUC) was used extensively to assess the particle assembly of the truncated version of the viral capsid protein as a vaccine antigen (Yang et al. 2013).

3.6 Newer VLP-Based Vaccines in Clinical Development

Given the success of the recombinant VLP approach to develop antiviral vaccines for HBV, HPV, HEV and influenza, this approach is likely to be applied to other viral vaccines where direct cell culture techniques are problematic. We will discuss here two such potential products which have advanced into the clinic and meet potential needs which otherwise could not be targeted by classical vaccine methods.

3.6.1 Chikungunya (CHIKV)

Chikungunya (CHIKV) is an arthropod-borne alphavirus (family *Togaviridae*). The alphaviruses are small enveloped positive-strand RNA viruses of 65–70 nm in diameter. This arboviral disease has been epidemic in Africa and parts of Asia with transmission occurring through *multiple* mosquito species. As of 2010 as reported by the Centers for Disease Control and Protection, more than 35 countries have documented cases of CHIKV infection excluding those countries where only imported cases have been documented (Ross 1956).

At present, there are no licensed vaccines or approved antiviral therapy currently available for the prevention or treatment of CHIKV infection. However, the US Department of Defense previously studied a live, attenuated vaccine for the prevention of chikungunya disease (Eckels et al. 1970). In that study, 98 % of vaccinees developed neutralizing antibodies after a single subcutaneous immunization, confirming feasibility for a vaccine for chikungunya disease. The Vaccine Research Center at the National Institute of Allergy and Infectious Diseases has developed a noninfectious VLP technology to induce robust immune responses for the prevention

of chikungunya infection (Akahata and Nabe 2012). The CHIKV VLPs developed by the NIAID contain the E1 and E2 glycoproteins that express the major antigen determinants and the capsid protein. The VLPs are produced by transient transfection of an HEK 293 cell line variant with a DNA plasmid encoding the structural genes of the CHIKV (capsid, E3, E2, 6K and E1, although the 6K and E3 proteins have not been specifically detected in the VLPs). The enveloped VLPs self-assemble and are released into the culture medium as approximately 65 nm particles. This vaccine entered clinical study under IND at the NIH Clinical Center through the VRC Clinical Trials Core.

3.6.2 Norovirus

Norovirus infection, more commonly known as the "winter vomiting disease", is the most common cause of nonbacterial acute gastroenteritis in the U.S., estimated by the CDC to afflict 23 million people per year (Mead et al. 1999). Recent studies from the CDC underscore the potential of norovirus infection to lead to severe complications, especially in children and vulnerable elderly individuals. A systematic review of studies that employed sensitive molecular assays for diagnosis revealed that norovirus causes an estimated, annual 64,000 hospitalizations and 900,000 clinical visits among children in industrialized nations and up to 200,000 deaths of children <5 years of age in developing countries (Patel et al. 2008).

Norovirus infection usually presents as acute-onset vomiting, watery nonbloody diarrhea with abdominal cramps, nausea, and fever. Dehydration is the most common complication, especially among the very young, elderly, and immunocompromised populations and frequently requires medical intervention including hospitalization. Such outbreaks are also a concern for the military where they interfere with combat readiness. Outbreaks are now often seen in special circumstances such as aboard large vessels or cruise ships.

Takeda Vaccines Inc. is developing a vaccine for the prevention of acute gastroenteritis caused by norovirus. The vaccine, Norovirus GI.1/GII.4 VLP (Norovirus Bivalent VLP) vaccine, consists of norovirus virus-like particles (VLP) formulated with aluminum hydroxide and monophosphoryl lipid A (MPL) adjuvants. The two VLPs in the vaccine formulation are a Norwalk virus VLP from genogroup GI.1 and a norovirus VLP consensus sequence from genogroup GII.4. The norovirus consensus VLP is a construct representing a consensus sequence from several norovirus GII.4 strains. The two VLPs in the vaccine are intended to provide broad protection against norovirus infections. The VLPs are produced in a baculovirus/insect cell expression system. A stable recombinant baculovirus encoding the norovirus VP-1 subunit is prepared in adherent Sf9 insect cells.

3.7 VLPs are Well-Characterized Vaccines

Based on the above discussed analytical methods, recombinant HPV VLP-based vaccines can be classified as "well-characterized vaccines," thus making it possible to use analytical characterization data (in addition to routine release data) to implement, after regulatory approval, a process change or scale-up without requiring a new clinical trial. It is important to have a comprehensive plan for comparability testing to support postlicensure scale-up, process improvement, and facility changes. A database is needed to capture the data based on the lots manufactured during process development, and particularly on the lots that had been used in the clinical trials. A weighted approach should be employed when analyzing the data, for instance, the potency assay or in vitro antigenicity analysis should carry more weight than a parameter such as VLP size. Maintaining a dynamic database on the process experience, on pre- and post-licensure lots, is critically important for the life cycle management of a marketed vaccine. Clearly, as new methods for characterizing proteins evolve, they should be applied to VLP vaccines. It would be critical to hold retains of pivotal production lots for this purpose.

Acknowledgement The authors would like to thank John A. Gilly, Leidos Biomedical Research, Inc. Vaccine Research Center (NIH) at Frederick and Charles R. Petrie, Takeda Vaccines, Inc. Bozeman, MT for contributing sections on Chikungunya and Norovirus, respectively.

References

Abbott_Diagnostics (2012) 40 years of hepatitis leadership. http://international.abbottdiagnostics.com/About_Us/Hepatitis_Leadership/

Akahata W, Nabe LG (2012) A specific domain of the Chikungunya virus E2 protein regulates particle formation in human cells: implications for alphavirus vaccine design. J Virol 86:8879–8883

Capen R, Shank-Retzlaff M, Sings H, Esser M, Sattler C, Washabaugh M et al. (2007) Establishing potency specifications for antigen vaccines. BioProcess Int 5:30–42

CHMP (2006) European medicines agency final report GARDASIL scientific discussion. EMEA

Cohen S, Ward G, Tsai P (1999) MALDI-MS characterization of human papillomavirus protein. Proceedings of the 47th American society for mass spectrometry conference, Dallas, TX, pp 13–17

Cohen S, Ward G, Oswald B, Tsai P (2000) A novel approach to analyze membrane proteins and peptides by mass spectrometry. Proceedings of the 48th American society for mass spectrometry conference, Long Beach, CA

Cox M (2011) A fast track influenza virus vaccine produced in insect cells. J Invertebr Pathol 107:531–541

Cox M (2012) Recombinant protein vaccines produced in insect cells. Vaccine 30:1759–1766

Descamps J, Giffroy D, Remy E, Mortiaux F, Mareschal J-C, Ponsar C et al. (2011) A case study of development, validation and acceptance of a non-animal method for assessing human vaccine potency. Procedia Vaccinol 5:184–191

Deschuyteneer M, Elouahabi A, Plainchamp D, Plisnier M, Soete D, Corazza Y et al (2010) Molecular and structural characterization of the L1 virus-like particles that are used as vaccine

antigens in Cervarix, the ASO4-adjuvanted HPV-16 and -18 cervical cancer vaccine. Human Vaccines 6:407–419
Dunne E, Datta S (2008) A review of prophylactic human papillomavirus vaccines: recommendations and monitoring in the US. Cancer 113:2995–3003
Eckels K, Harrison V, Hetrick F (1970) Chikungunya virus vaccine prepared by tween-ether extraction. Appl Microbiol 19:321–325
EP (2008) Hepatitis B vaccine (rDNA) 01/2008:1056. In: EP, European Pharmacopea, EDQM
EP (2010a) Human papillomavirus vaccine (rDNA) 01/2010:2441. In: European Pharmacopeia, EDQM
EP (2010b) Assay of hepatitis B vaccine 01/2008:20715. In: European Pharmacopea, EDQM
FDA (2013) http://www.flublok.com/pscp2.pdf
Fischman J (2006) Sticking it to cancer. US news and world report
Fox C (2012) Characterization of TLR4 agonist effects on alhydrogel(R) sedimentation: a novel application of laser scattering optical profiling. J Pharm Sci 101:4357–4364
Gavilanes F, Gonzalez-Ros JM, Peterson DL (1982) Structure of hepatitis B surface antigen. J Biol Chem 257:7770–7777
Gavilanes F, Gomez-Gutierrez J, Miguel Gonzalez-Pos J, Ferragut J, Guerrero E et al (1990) Hepatitis B surface antigen role of lipids in maintaining the structural and antigenic properties of protein components. Biochem J 265:857–864
Gilbert R, Beales L, Blond D, Simon M, Lin B, Chisari F et al. (2005) Hepatitis B small surface antigen particles are octahedral. Proc Natl Acad Sci USA 102:14783–14788
Grachev VP, Magrath DI (1993) Quality control of hepatitis B vaccine. In: Ellis RW (ed) Hepatitis B vaccines in clinical practice. Dekker, New York, pp 103–121
Greiner V, Egele C, Oncul S, Ronzon F, Manin C, Klymchenko A et al (2010) Characterization of the lipid and protein organization in HBsAg viral particles by steady-state and time-resolved fluorescence spectroscopy. Biochimie 92:994–1002
Guha S, Li M, Tarlov MJ, Zachariah MR (2012) Electrospray–differential mobility analysis of bionanoparticles. Trends Biotechnol 291–300
Harrison S (1990) Fields virology. In: Fields virology, vol 2. Raven, New York, pp 37–61
Hemling ME, Carr SA, Capiau C, Petre J (1988) Structural characterization of recombinant hepatitis B surface antigen protein by mass spectrometry. Biochemistry 27:699–705
Hilleman M (1993) Plasma-derived hepatitis B vaccine: a breakthrough in preventive medicine. In Ellis RW (ed) Hepatitis B vaccines in clinical practice. Dekker, New York, pp 17–39
Le Duff Y, Blanchet M, Sureau C (2009) The pre-S1 and antigenic loop infectivity determinants of the hepatitis B virus envelope proteins are functionally independent. J Virol 3:12443–12451
Le Tallec D, Doucet D, Ekouahabii P, Deschuyteneer M, Deschamps M (2009) Cervarix, The GSK HPV-16/HPV-18 AS04-adjuvanted cervical cancer vaccine, demonstrates stability upon lon-term storage and under simulated cold chain break conditions. Human Vaccines 5:467–474
Li Y, Bi J, Zhao W, Huang Y, Sun L, Zeng A-P et al (2007) Characterization of the large size aggregation of hepatitis B virus surface antigen (HBsAg) formed in ultrafiltraion process. Process Biochem 42:315–319
Li S, Tang X, Seetharaman J, Yang C, Gu Y et al. (2009) Dimerization of hepatitis E virus capsid protein E2 s domain is essential for virus–host interaction. PLoS Pathog 5(8):e1000537
Mach H, Volkin D, Troutman R, Wang B, Luo Z, Jansen K et al (2006) Disassembly and reassembly of yeast derived recombinant human papillomavirus-like particles (HPV VLPs). J Pharm Sci 95:2195–2206
MacNair JEDT (2005) Alignment of absolute and relative molecular size specifications for a polyvalent pneumococcal polysaccharide vaccine (PNEUMOVAX 23). Biologicals 33:49–58
Mangold C, Unckell F, Wer RM, Streeck R (1995) Secretion and antigenicity of hepatitis B virus small envelope proteins lacking cysteines in the major antigenic region. Virology 211:535–543
Markowitz L, Hariri S, Lin C, Dunne E, Steinau M, McQuillan G et al (2013) Reduction in human papillomavirus (HPV) prevalence among young women following HPV vaccine introduction in the United States, national health and nutrition examination surveys, 2003–2010. J Infect Dis 208:385–393

Mead P, Slutsker L, Dietz V, McCaig L, Bresee J, Shapiro C et al (1999) Food-related illness and death in the United States. Emerg Infect Dis 607–625

Milne J, Borgnia M, Bartesaghi A, Tran E, Earl L, Schauder D et al (2013) Cryo-electron microscopy–a primer for the non-microscopist. FEBS J 280:28–45

Mohr J, Chuan Y, Wu Y, Lua L, Middelberg A (2013) Virus-like particle formulation optimization by miniaturized high-throughput screening. Methods 60:248–256

Mulder A, Carragher B, Towne V, Meng YW, Dieter L, Potter C et al. (2012) Toolbox for non-intrusive structural and functional analysis of recombinant VLP based vaccines: a case study with hepatitis B vaccine. PLos One 7:e33235

Murphy G, Jensen G (2007) Electron cryotomography. Biotechniques 43:413

Orlova E, Saibil H (2011) Structural analysis of macromolecular assemblies by electron microscopy. Chem Rev 111:7710–7748

Patel M, Widdowson M, Glass R, Akazawa K, Vinje J, Parashar U (2008) Systematic literature review of role of noroviruses in sporadic gastroenteritis. Emerg Infect Dis 2008:1224–1231

PDA (2012) A-VAX: applying quality by design to vaccines. http://www.ispe.org/2013-biotechconference/a-vax-applying-qbd-to-vaccines.pdf

Pease LF, Lipin D, Tsai D-H, Zachariah M, Lua L, Tarlov M et al (2009) Quantitative characterization of virus-like particles by asymmetrical flow field flow fractionation, electrospray differential mobility analysis, and transmission electron microscopy. Biotechnol Bioeng 102:845–855

Peterson DL (1981) Isolation and characterization of the major protein and glycoprotein of hepatitis B surface antigen. J Biol Chem 256:6975–6983

Peterson DL, Nath N, Gavilanes F (1982) Structure of hepatitis B surface antigen correlation of subtype with amno acid sequence and locatio of the carbohydrate moeity. J Biol Chem 257:10414–10420

Petre J, Van Wijnendaele F, De Neys B, Conrath K, Van Opstal O, Hauser P et al (1987) Development of a hepatitis B vaccine from transformed yeast cells. Postgrad Med J 63(Suppl 2):73–81

Ross R (1956) The Newala epidemic. III. The virus: isolation, pathogenic properties and relationship to the epidemic. J Hyg 54:177–191

Salisse J, Sureau C (2009) A function essential to viral entry underlies the hepatitis B virus a determinant. J Virol 83:9321–9328

Schofield T (2002) In vito versus in vivo concordance: a case study of the replacement of an animal potency test with an immunochemical assay. In: Karger BF (ed) Advancing science and elimination of the use of laboratory animals for development and control of vaccines and hormones. Karger, Basel, pp 299–304

Shank-Retzlaff M, Wang E, Morley T, Anderson C, Hamm M, Brown M et al (2005) Correlation between mouse potency and in vitro relative potency for human papillomavirus type 16 virus like particles and gardasil vaccine samples. Human Vaccines 1:191–197

Shank-Retzlaff M, Zhao Q, Anderson C, Hamm M, High K, Nguyen M et al (2006) Evaluation of the thermal stability of gardasil. Human Vaccines 2:147–154

Shi L, Sings H, Bryan J, Wang B, Wang Y, Mach H et al (2007) GARDASIL: prophylactic human papillomarvirus vaccine development—from bench top to bed-side. Clin Phamacol Ther 81:259–264

Short J, Chen S, Roseman A, Butler P, Crowther RA (2009) Structure of hepatitis B surface antigen from subviral tubes determined by electron cryomicroscopy. J Mol Biol 390:135–141

Sitrin R (2010) After the license approval: how analytics can keep you in the market. In: Vaccine technology III, Nuevo Vallarta, Mexico: engineering conferences international, http://dc.engconfintl.org/cgi/viewcontent.cgi?article=1028&context=vaccine_iii

Sitrin RD, Wampler DE, Ellis RW (1993) Survey of licensed hepatitis B vaccines and their production processes. In: Ellis RW (ed) Hepatitis B vaccines in clinical practice. Dekker, New York, pp 83–102

Stephenne J (1990) Development and production aspects of a recombinant yeast-derived hepatitis B vaccine. Vaccine 8 suppl:S69-S73

Stirk H, Thornton J, Howard C (1992) A topological model for hepatitis B surface antigen. Intervirology 33:148–158

Towne V, Zhao Q, Brown M, Finnefrock A (2013) Pairwise antibody footprinting using suface plasmon resonance technology to characterize human papilloamavirus type 16 virus like particles with direct anti-HPV antibody immobilization. J Immunol Methods 388:1–7

Wampler DE, Lehman ED, Bodger J, McAleer WL, Scolnick EM (1985) Multiple chemical forms of hepatitis B surface antigen produced in yeast. Proc Nat Acad Sci 82:6830–6834

WHO (1988) Requirements for hepatitis B vaccine prepared from plasma. Requirements for biological substances 31. World health organization technical report series 771; annex 8, pp 181 = -207

WHO (1989) Requirements for hepatitis B vaccines made by recombinant DNA techniques. WHO, Requirements for biological substances no 45. World health organization, technical report series, no 786, pp 38–70

WHO (2006) Guidelines to assure the quality, safety and efficacy of recombinant papillomavirus virus-like particle vaccines. Expert comitttee on biological standardization WHO/BS/06.2050

Yang C, Pan H, Wei M, Zhang X, Wang N, Gu Y et al (2013) Hepatitis E virus capsid protein assembles in 4M urea in the presence of salts. Protein Sci 22:314–326

Zhang J, Gu S, Li S, He Z, Huang G, Zhuang H et al (2005) Analysis of hepatitis E virus neutralization sites using monoclonal antibodies directed against a virus capsid protein. Vaccine 23:2881–2892

Zhao Q, Wang Y, Freed D, Fu T-M, Gimenez J, Sitrin R et al (2006) Maturation of recombinant hepatitis B surface antigen particles. Human Vaccines 2:174–180

Zhao Q, Towne V, Brown M, Wang Y, Abraham D, Oswald CB et al (2011a) In-depth process understanding of RECOMBIVAX HB maturation and potential epitope improvements with redox treatment: multifaceted biochemical and immunochemical characterization. Vaccine 29:7936–7941

Zhao Q, Wang Y, Abraham D, Towne V, Kennedy R, Sitrin R (2011b) Real time monitoring aof antigenicity development of HBsAg virus like particles (VLPs) during heat- and reox-treatment. Biochem Biophys Res Commun 408:447–453

Zhao Q, Allen M, Wang Y, Wang B, Wang N, Shi L et al (2012a) Disassembly and reassembly improves morphology and thermal stability of human papillomavirus type 16 virus like particles. Nanomed Technol Biol Med 8:1182–1189

Zhao Q, Modis Y, High K, Towne V, Meng Y, Alexandrof J et al (2012b) Disassembly and reassembly of human papillomavirus virus like particle produces more virion like antibody activity. Virol J 9:1–13

Zhao Q, Jun Z, Jun Wu T, Li S-W, Ng M-H et al (2013a) Antigenic determinants of hepatitis E virus and vaccine-induced immunogenicity and efficacy. J Gastroenterol 48:159–168

Zhao Q, Li S, Yu H, Xia N, Modis Y (2013b) Virus-like particle-based human vaccines: quality assessment based on structural and functinal properties. Trends Biotechnnol 31:654–663

Zhao Q, Potter C, Carragher B, Alexandroff J, Towne V, Abraham D et al (2014) Use of cryo electron microscopy to visualize the structural features and binding to functional antibodies of virus-like particles in GARDASIL®. Human Vaccines Immunother 10:734–739

Zhu F, Zhang J, Zhang X, Zhou C, Wang Z, Huang S et al (2010) Efficacy and safety of a recombinant hepatitis E vaccine in healthy adults: a large-scale, randomised, double-blind placebo controlled, phase 3 trial. Lancet 376(9744):895–902

Chapter 4
Analysis of Influenza Vaccines

Pierre-Alain Moisset, Jeffrey Pederson and Nathalie Landry

4.1 Introduction

Influenza is a seasonal illness that occurs mostly in the winter periods, from October to March in the Northern hemisphere and from May to September in the Southern hemisphere (World Health Organization 2012b). Approximately 3–5 million cases of severe illness and 250,000–500,000 deaths occur every year worldwide, generating costs of billions of dollars (influenza and influenza-like illness) (World Health Organization 2009).

Vaccination has been widely accepted as an important defense against the spread of influenza virus. Protection levels of approximately up to 70–90 % against clinical disease in healthy adults have been reported in industrialized countries, provided there is a good match between the vaccine and the circulating strains (Nichol 2008). A recent meta-analysis of 31 clinical trials revealed that the pooled vaccine efficacy was 59 % in the case of drifted strains (Osterholm et al. 2012).

The yearly production of quality-controlled flu vaccines in adequate amounts requires manufacturers to overcome a significant number of hurdles. Before the first dose of seasonal vaccine is produced and distributed, many steps have to be followed by all manufacturers and although they can influence the decision process, many parameters are not under their control as outlined in the figure and sections below (Fig. 4.1).

P.-A. Moisset (✉)
GlaxoSmithKline, 2323, Boul. Du Parc Technologique, Quebec, Quebec G1P 4R8, Canada
e-mail: pierre-alain.a.moisset@gsk.com

J. Pederson
KBI Biopharma, Inc, 1101 Hamlin Road, Durham NC 27704, USA

N. Landry
Medicago, 1020, Route de L'Église, Bureau 600, Québec G1V 3V9, Canada

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6_4

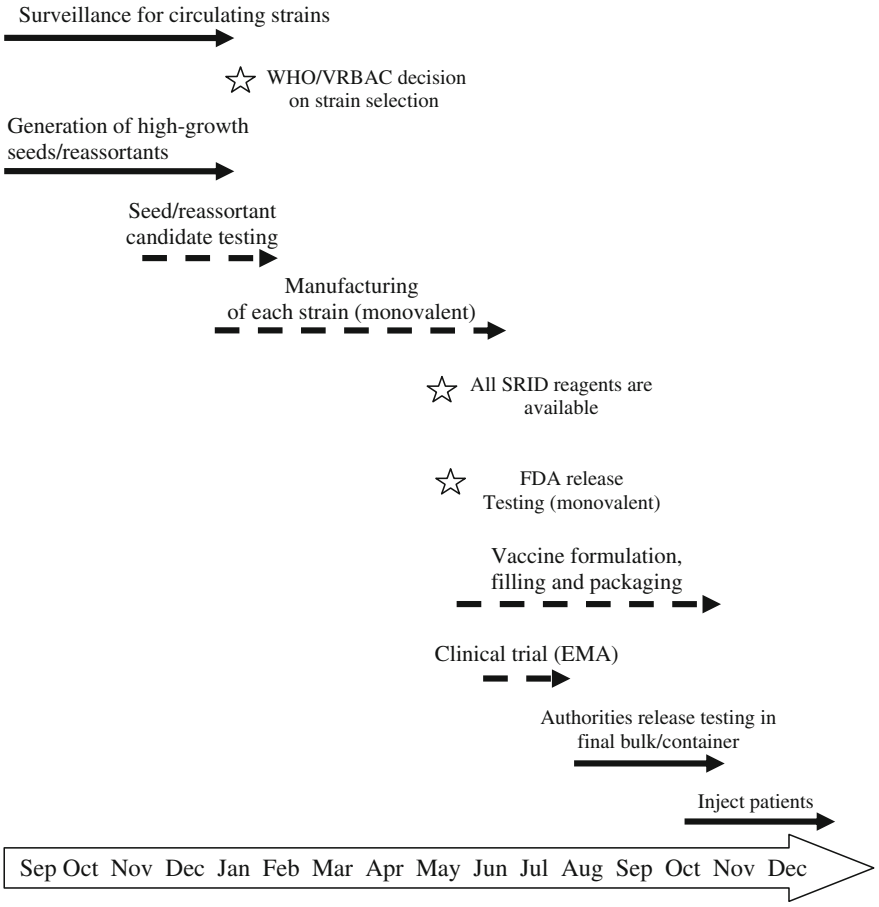


Fig. 4.1 Timeline for influenza vaccine manufacturing in the Northern hemisphere (*full lines* represent steps performed by authorities, *dashed lines* represent steps performed by the manufacturers)

4.2 Influenza Vaccine Basics: Hemagglutinin and Neuraminidase

There are three types of influenza viruses, A, B, and C which can all infect humans (Young 2000). As influenza A and B strains cause the most disease and outbreaks in the world, including pandemics, these types have been included in the composition of the vaccine for the last 40 years.

The common constituents of influenza vaccines are the most abundant surface glycoproteins from influenza A and B: hemagglutinin (HA) and neuraminidase (NA).

Influenza A viruses are divided into subtypes based upon the HA and NA glycoproteins. Seventeen different subtypes of HA and nine subtypes of NA are known today (Tong et al. 2012). Only the most prevalent subtypes of HA (H1, H2, and H3) and of NA (N1, N2) identified in the human population have been included in the vaccines. Influenza B strains also possess HA and NA glycoproteins. However, they are not differentiated into subtypes but rather in two antigenically distinct lineages, B/Yamagata and B/Victoria. As both lineages have been cocirculating in the population, they have both been included in the vaccine formulations over the past years.

Virus subtypes differ serologically and antibodies to one subtype do not recognize other subtypes. Influenza viruses have a high mutation rate and consequently, the antigenic properties of circulating influenza strains are constantly evolving. Two different mechanisms drive change in influenza viruses; antigenic drift and antigenic shift. Antigenic drift is caused by point mutations in genes that alter antigenic properties. For influenza, these point mutations can occur in the HA and NA genes, which result in minor changes to the amino acid sequence on these proteins. In turn, these changes in amino acid sequence may allow the "new" virus to escape detection by the immune system. These variants with new antigenic properties have a selective advantage if these amino acid substitutions allow escape from the immune system. Antigenic shifts are caused by an exchange of RNA between different viruses that coinfect a host. The result is a drastic change in the virus antigenic properties, creating a virus to which no preexisting immunity exists in the population. Antigenic shifts are the major cause of Influenza pandemics.

Influenza A infects a wide range of species such as birds and mammals and can change by both antigenic drift and shift, thus posing a much higher concern than B strains for the cause of pandemics. Alternatively, influenza B infects almost exclusively humans, has a threefold lower mutation rate than A strains and evolves only by antigenic drift (Hay et al. 2001).

Because of the evolving properties of HA and NA, the strains included in influenza vaccine formulations need to be revised every year. Indeed, as strains constantly change by antigenic drift, the efficacy of the vaccine gradually decreases and formulations containing the most updated version of the HA and NA circulating at a given moment need to be manufactured. The influenza activity is constantly monitored around the globe by National Influenza centers and recommendations for vaccine formulations are emitted twice a year by the World Health Organization (WHO), one for the Northern Hemisphere and another for the Southern Hemisphere.

4.3 Egg-Based Manufacturing Process

Vaccination is a safe and cost-effective way to prevent influenza infections. Influenza vaccines have been manufactured using egg-based processes for the last 70 years. Two different types of vaccines have been manufactured: live attenuated

influenza vaccines (LAIV) and inactivated influenza vaccines (whole, split, or subunit vaccines). As explained in the sections below, both live and inactivated vaccines go through the same two initial steps before the actual manufacturing process takes place, the reassortment and egg adaptation processes.

4.3.1 Reassortment Process

Each year, based on the available epidemiological data, WHO recommends the virus strains to be included in the next vaccine formulation. The selection process is complex and will not be covered in detail here.

Generally, the strains that are selected for inclusion in upcoming vaccine formulation do not grow well in eggs. The objective of the selection process is to identify strains that grow to high titers in eggs or to generate reassortant influenza strains having high growth properties. Reassortant strains contain the same essential influenza proteins needed to induce immunity as their wild-type counterparts, HA and NA, but also viral proteins from another influenza virus which allows them to grow to higher titers in eggs.

The classical approach to creating reassortants involves co-infection of two different virus strains in eggs to produce a mixed progeny containing genes from both the wild-type and donor strains. For A strains, this is usually done by coinfecting the wild-type virus of interest with a select high-growth, attenuated laboratory strain such as A/PR8/34 (H1N1), A/Okuda/57 (H2N2) or the cold-adapted A/Ann Arbor/6/60 (H2N2), A/Leningrad/134/57 (H3N2) in chicken eggs (Wareing and Tannock 2001). A subsequent selection process results in a hybrid virus that contains HA and NA proteins of the wild-type strain and "backbone genes" from the high-growth donor virus (Kilbourne 1969). For B strains used in inactivated vaccines, the reassortment process is generally not used because a high-growth laboratory strain that can be easily isolated from the progeny viruses is not yet available. Reassortment in B strains is performed for the LAIV using cold-adapted B/Ann Arbor/1/66 and B/USSR/60/69 (Wareing and Tannock 2001).

The second approach, reverse genetics, uses cell lines, cloned viral DNA with or without helper virus to produce reassortants (Engelhardt 2012). Many variations of reverse genetics have been published, but the most useful method uses only cDNA and helper cell lines because it allows the full control on the combination of the eight segments of viral RNA included in the reassortant without any need for a selection process (Fodor et al. 1999; Neumann et al. 1999). Nonetheless, because of its complexity, this approach is currently used mainly for the production of pandemic reassortants (see below) or for research purposes, and not for seasonal production.

4.3.2 Egg Adaptation Process

The egg adaptation process allows influenza strains to grow to higher titers than their wild-type counterparts (Robertson et al. 1993; Gambaryan et al. 1999). In human clinical isolates, HA is responsible for the attachment of the virus to its host receptor. HA contains amino acids sequences which are "optimized" for human cell receptors (α2,6 sialosides), but do not allow the virus to attach and replicate efficiently in eggs. This is especially true with B viruses. When these isolates are passaged into the egg allantoic cavity, key amino acid sequences were shown to selectively change by substitution, leading most often to the loss of a glycosylation site in the receptor-binding site of HA and increased affinity for egg receptors, the α2,3 sialosides (Robertson et al. 1993; Gambaryan et al. 1997). The impact of egg adaptation on the match between the circulating strain (or isolate) and the egg-grown virus is not well known (Dormitzer et al. 2012).

Successive passages can also produce other mutations affecting the HA amino acid sequence without necessarily impacting the receptor-binding functions. It has been reported to sometimes impact the potency assay (Rodda et al. 1981).

4.3.3 Seasonal Production of Reassortants Influenza Strains: Variants and Yields

The hemagglutination inhibition assay (HAI) and microneutralization (MN) are two commonly used tests which can detect subtle differences in the antigenic properties of hemagglutinin (World Health Organization 2012b). Using specific antisera against HA, these assays allow segregating influenza strains into clusters of antigenically similar HA. These tests, in addition to genotyping, are crucial in the WHO decision-making process for the inclusion of strains in seasonal vaccines.

Although some isolates or reassortants can share antigenic similarities in their respective hemagglutinin and neuraminidase, they can nevertheless have different growth properties in eggs (Robertson et al. 1994). In order to produce efficiently the required number of doses, manufacturers carefully choose the variant that is antigenically indistinguishable from the wild-type strain but has high-growth properties.

A critical consideration when evaluating high-growth reassortants is the availability of reference reagents. Among the many high-growth reassortants or variants that are generated for each recommended strain, only a selected few are chosen for the production of reference reagents (Minor 2010). The selection process is a collaborative effort between manufacturers and the laboratories responsible for producing the reagents. In a short period of time, manufacturers need to evaluate the candidate reassortants, share data, and determine which strains should be used for the production of reference reagents. Four countries, namely the USA, United Kingdom, Australia, and Japan use a similar decision-making process and have their respective laboratories for reagent production, CBER, NIBSC, TGA, and NIID.

4.3.4 Pandemic Production

The outbreak of a pandemic requires manufacturing of massive amounts of vaccines to supply world demand in a very short period of time (Friede et al. 2011). The same steps described in the above sections for seasonal vaccine manufacturing also apply to a pandemic but at an accelerated pace to limit the extent of the outbreak. Pandemic preparedness encompasses a series of initiatives explored during the interpandemic period to optimize the manufacturing and supply processes (Kieny et al. 2006). Initiatives related to the analysis of the virus are discussed below:

Potential pandemic strains are closely monitored worldwide by the WHO surveillance network, using the same analytical tools described above for seasonal strains. The genetic, pathogenic, and antigenic profiles of the isolates are established and they are segregated into subtypes. Nonpathogenic reassortants are produced from the most representative variants of each subtype (discussed further below).

In an effort to increase knowledge on the manufacturability and on the clinical potential of these variants, manufacturers and authorities have been collaborating to produce limited amounts of different prepandemic vaccines for clinical studies and/or stockpiling (prepandemic manufacturing) (Abelin et al. 2011). Alternative methods are being explored to evaluate HA concentration in vaccine (discussed below). However, to allow the full characterization and release of prepandemic vaccines, reference reagents must be produced and distributed by at least one of the four reagent producing laboratories.

Throughout the years, experience has shown that prepandemic and pandemic strains can indeed be manufactured with the same established processes used for seasonal strains (Hehme et al. 2002; Stephenson et al. 2003; Hehme et al. 2004). Nonetheless, clinical studies have also shown that higher doses can be sometimes required to elicit an adequate immune response for pandemic strains (Treanor et al. 2001). This, added to the fact that a pandemic outbreak would result in an overwhelming demand for vaccine, led to the implementation of dose-sparing strategies and new vaccine formulations to improve immunogenicity (Nichol and Treanor 2006). Consequently, the main potency assay, the single radial immunodiffusion assay (SRID) was adapted and revalidated to take into account novel formulations (further discussed in sections below).

Some isolates of potential pandemic strains are pathogenic for humans and poultry, a situation that precludes the use of the classical reassortment method and egg-based manufacturing in standard conditions. For example, influenza virus of the H5 and H7 serotypes contains a polybasic cleavage site in the hemagglutinin (HA) protein. This polybasic cleavage site is the main virulence factor for these avian strains as it allows for proteolytic HA activation by the ubiquitous protease furin, resulting in systemic viral spread and lethality. By contrast, the HA from low pathogenic avian influenza strains (LPAIs) is cleaved by tissue-restricted proteases with monobasic specificity, resulting in mild disease or even subclinical infection

(Alexander 2000; Suarez 2010; Veits et al. 2012). Highly pathogenic influenza (HPAI) strains cannot be produced in embryonic eggs without proper adaptation as they kill the embryo before the vaccine can be produced. To overcome this, a small genetic modification of the isolates HA sequence has to be made prior to virus infection in egg-based manufacturing. The molecular modification is done ex vivo so reverse genetics is used to produce the reassortant. For example, the reference H5N1 vaccine (NIBRG-14), provided from the UK National Institute for Biological Standards and Control (NIBSC), is a reassortant virus containing NA and a HA gene mutated in the polybasic cleavage site region segments of A/Vietnam/1194/2004 (H5N1) virus and the other six gene segments of egg-adapted high growth A/PR/8/1934 (H1N1) virus (Nicolson et al. 2005). This technology was shown to greatly improve the readiness to an eventual pandemic by reducing the lead time to available reassortants. However, the reassortant strains developed for highly pathogenic pandemic strains contain more mutations in the surface HA glycoprotein than reassortants produced for seasonal strains, especially in the hemagglutinin cleavage site or in the surface neuraminidase protein (Bogs et al. 2010; Zhang et al. 2011).

However, one issue to be resolved is that helper cell lines used in reverse genetics are patented, thus requiring to solve licensing and intellectual property issues before commercial manufacturing (Webby et al. 2004).

4.4 Novel Production Systems

4.4.1 Cell Culture Based

Most current seasonal influenza vaccines are manufactured using chicken embryonated eggs, which is labor-intensive and hard to scale up during a pandemic. Pandemic influenza strains are often pathogenic to eggs and require adaptation before vaccine production can be initiated and this process delays the availability of vaccines. Moreover, egg supply may not be available during a pandemic due to H5N1 viruses that are highly pathogenic to chickens. Therefore, the WHO has been encouraging the development of cell-based influenza H5N1 vaccines since 2006 (World Health Organization 2006). Two cell lines, Vero and Madin-Darby Canine Kidney (MDCK) cells, have been licensed for manufacturing influenza vaccines (Kistner et al. 1998; Doroshenko and Halperin 2009). In addition to influenza vaccines, Vero cells have been widely approved for manufacturing other human vaccines, but MDCK cells are only licensed for influenza vaccines. MDCK cells are used for the production of a trivalent seasonal vaccine by Novartis.

4.4.2 Other Technologies

Other technologies have been developed that do not rely on the reassortment or reverse genetic processes. These technologies do not rely on the growth of influenza viruses but rather use genetic sequence or production of recombinant antigens. The production of novel influenza vaccines is made in insect cell culture using the baculovirus expression technology, in *E. coli* or in plants. Vaccines made by these alternative manufacturing technologies have been tested in clinical trials and one candidate vaccine, FluBlok® has recently received FDA approval. Table 4.1 summarizes the development status of these new vaccines. As they are produced by novel manufacturing technologies, the use of reference standard potency assays pose some challenges discussed in the following sections.

Table 4.1 Status of influenza vaccines made by alternative production technologies by end of 2012

Manufacturing technology	Vaccine type	Manufacturer	Status
Insect cells	Recombinant HA	Protein sciences, USA	Seasonal: approved pandemic: phase II completed
Insect cells	Virus-like particle	Novavax, USA	Pandemic: Phase I ongoing (NCT01594320 and NCT01596725) seasonal: phase II completed (NCT00903552)
MRC5 cells	Live-Ad4-vectored vaccines	Pax Vax	Pandemic: phase 1 completed (WHO 2012)
E. coli	M2 protein with flagellin	Vaxinnate corp.	Seasonal: phase I/II completed with seasonal vaccine (PLOS ONE 2010, 5, Issue 12, e1442)
E. coli	DNA vaccine	NIH USA and CSL Australia	Seasonal: phase I completed (NCT00995982)
E. coli	DNA vaccine	NIH USA and Sanofi Pasteur	Seasonal: phase Ib completed (NCT01498718, NCT1609998) pandemic: phase I completed (Lancet Infect. Dis. 2011 11(12), 916–924)
E. coli	DNA vaccine	NIH USA and GSK and CSL	Seasonal: phase I completed (NCT008558611)
E. coli	DNA vaccine	NIH USA and Novartis	Seasonal: phase I completed (NCT00973895)
Plants	Recombinant HA	Fraunhofer USA	Seasonal H1N1: phase I completed (NCT01177202)
Plants	Virus-Like Particle	Medicago Inc.	Seasonal H1N1: phase 1 completed (NCT01302990) Pandemic: phase II completed (NCT01244867)

4.5 The Influenza Vaccine Potency Assay: SRID

The analytical control strategy for flu vaccines is designed to evaluate safety, purity, identity, and potency. The control strategy involves substantial testing at numerous points in the production process (in-process) as well as bulk lot release and final product testing. This chapter will focus mainly on testing at four stages of production: viral seed banks, monovalent virus pool, final bulk, and final drug product. Certainly, the production system used to manufacture the vaccine must be considered when defining the number and type of tests required.

Identity testing is typically performed on viral seed banks, at the monovalent virus pool stage and on final drug product. Haemagglutinin (HA) content (SRID), HA-inhibition tests, neuraminidase inhibition assays (NA), or other immunoassays using specific antibodies can serve as identity tests.

Safety tests are numerous and are performed throughout the manufacturing process. Tests are performed to demonstrate that the virus has been effectively inactivated. This can be done at various steps of the process (typically at the monovalent bulk release and final bulk release steps) using egg or cell substrates. Adventitious agents' testing is done at different stages of the process. Both in vivo and in vitro testing can be used to detect a wide variety of potential adventitious agents. Although culture methods are still widely used, specific PCR assays have been developed to detect specific viral or microbial agents (e.g., adenoviruses, retroviruses, mycoplasma). The type and scope of the adventitious agents' testing strategy is very dependent on the production system being used. The typical bioburden, sterility, and endotoxin tests are performed at different steps in the process as well as general safety testing on final product.

Purity testing is also highly dependent on the production system. Purity tests will typically include protein assays such as SDS-PAGE and others, residual nucleic acid tests, and host-cell proteins. Purity evaluation will also include tests for process-related residues such as tests for chemicals used in the virus inactivation step.

Manufacturing yield can be monitored by different tests. The hemagglutination assay is widely used to evaluate the relative levels of hemagglutinin present in a sample (Killian 2008). It is a quick and simple assay that can be used with live and inactivated samples. However, no correlation between results from this method and HA potency assays can be established and no comparison between virus strains can be made as each virus strain has its own agglutination properties.

The infectivity of a live sample can be an early indicator of the yield of a manufacturing process. A high-growth reassortant is expected to replicate effectively in eggs or cell culture and thus to generate higher infectious titers. Infectivity is determined by three different assays: plaque assays, egg-infective dose 50 (EID50), or tissue culture infective dose (TCID50). Both EID50 and TCID50 assays allow calculating the amount of infectious particles by infecting the substrate (eggs or tissue culture) with serial dilutions of the sample. Alternatively, plaque assays are based on the ability for a virus to form plaques in a monolayer of cell culture overlaid with agarose (Klimov et al. 2012).

The established potency assay for flu vaccines is the SRID assay. The SRID assay is discussed in the following sections. Others assays used to measure potency or content include the NA assay and Hemagglutination titer assay.

4.5.1 Critical Assay Parameters

The SRID has been used as a potency assay for influenza vaccines for over three decades (Wood et al. 1977). The assay involves the incorporation of strain-specific antibodies into molten agar, poured into a template, and allowed to set. After the gel sets, wells are perforated that will be used for the addition of the test sample. Sample and standards are diluted, detergent is added, and the samples loaded into the wells. Samples and standards diffuse into the gel and a reaction of the antigen with the antibody produces a zone of precipitation. This precipitation occurs at the specific antibody to antigen ratio. Following a period of incubation, drying, and staining, the agar plate shows a series of concentric circles (Fig. 4.2). By using a reference antigen of known concentration, a standard curve of zone size can be generated and the potency of the sample determined relative to the reference antigen. The diameter of the precipitation ring is proportional to the logarithm of antigen concentration. Reference standard antigen and antibodies are obtained from WHO essential reference laboratories (CBER, NIBSC, TGA and NIID). When developing an SRID assay for flu potency determination, there are a number of critical assay parameters that need to be considered. These critical parameters include the type of gel used, data capture and evaluation, use of statistics, reagents used, plate design, and type of calculation.

One of the first considerations is how to pour the gel. In-house fabricated gel apparatus designed specifically for this application can be utilized. Another option is commercially available gel pouring templates such as GelBond. GelBond films are a transparent, flexible polyester films designed to support agarose gels. Regardless of the apparatus used to pour the gels, the end result must be a homogeneous gel of uniform thickness. The thickness and uniformity of the gels will impact diffusion of the antigen into the gel and hence, can impact the potency result generated. Adherence of the gel to the support surface is also important as air bubbles can impact diffusion of samples and standards. Air bubbles or uneven surface adhesion of the gel can cause difficulties when generating sample wells.

Another critical parameter that impacts assay performance is the choice of instrument for data capture. SRID is dependent upon the ability to measure accurately the diameter of the precipitation zones. This is traditionally done manually with the use of a ruler or calibrated viewer. Manual data capture is very laborious, time-consuming, and a contributing factor to assay variability. In addition to the time it takes to measure the precipitation ring, the data will then need to be manually entered into a software application for analysis. When speed to market is critical, manual data capture becomes a bottleneck and is not a preferred method of data capture. It is also a compliance risk to rely on the manual input of data. Automating the data capture aspect of the SRID assay is critical to improving

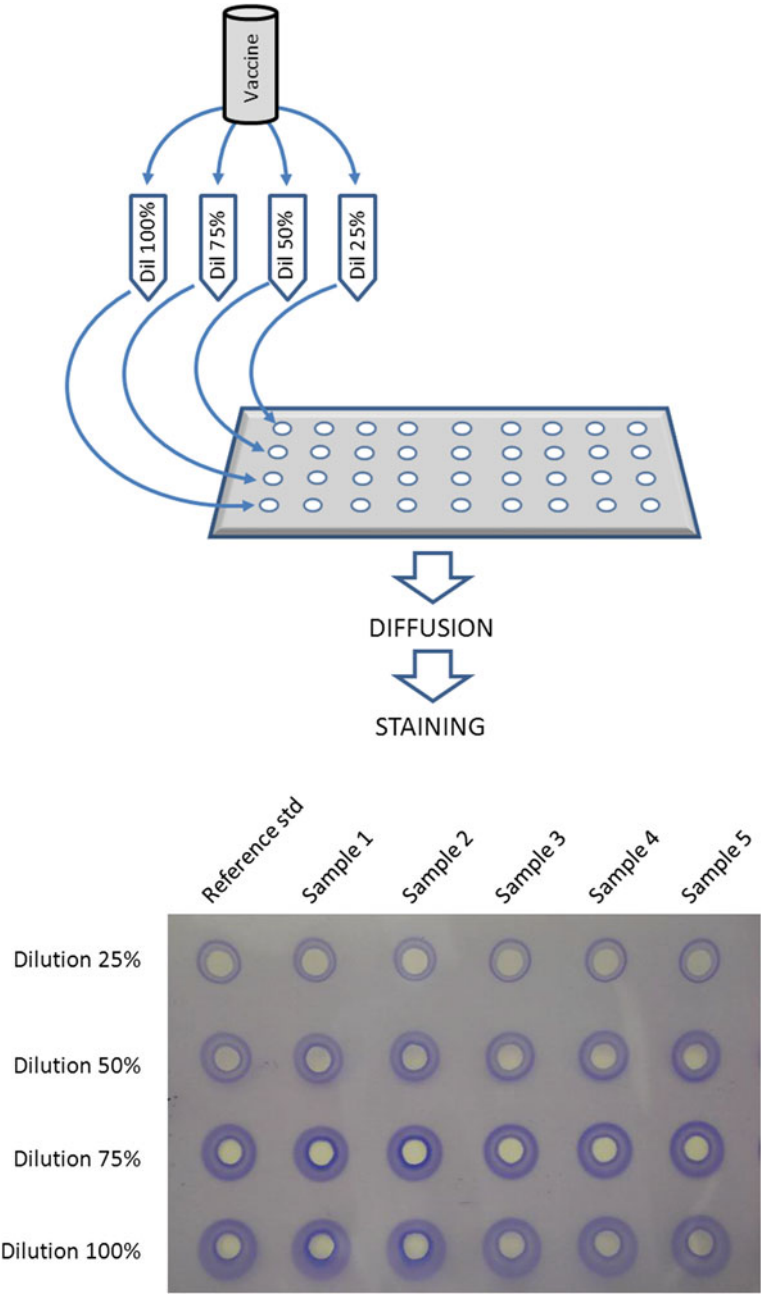


Fig. 4.2 SRID assay showing increasing amounts of antigen within each lane and the corresponding increase in *circle diameter*

productivity and compliance. There are several automation options available. A densitometer can be used to provide a reliable mechanism to measure ring diameter. Depending on the sharpness of the precipitation ring, the densitometer does not provide a completely automated process. Manual adjustment is generally required to fit an outline to the edge of the precipitation edge. Data analysis occurs using a separate software application. The ProtoCol System from Synbiosis provides another automation option. The ProtoCol system provides accurate zone measurements for many strains in seconds. Challenges still exist for the accurate measurement of zones from certain strains. Sharpness of the precipitation ring is dependent upon strain type and also the type of production system (i.e., any production system other than the traditional egg-based system). The system does flag "fuzzy" edges and allows manual adjustment. Imaging technology is used to capture the image and the detected area is converted to a diameter value. Essentially, an infinite number of measurements are made to generate the diameter value. Data is directly transferred into a statistical analysis program without the need to re-enter the information.

As the dose–response curve is nonlinear with SRID, two types of calculations are mostly used to approximate the HA content: the slope–ratio model or the parallel-line assay. The slope–ratio model uses the assumption that there is a common intercept between the reference and test sample. To calculate HA potency, the slope of the test sample is divided by the slope of the reference antigen and then multiplied by the reference potency value (Williams 1993). As this calculation method is simple, it is widely used worldwide. However, obtaining common intercepts between the product and reference antigen is not always feasible and the method is considered less robust than the parallel-line assay (van Kessel et al. 2012). Parallel-line assay (PLA) allows calculating potency in samples which do not share common intercepts. However, PLA requires that the dose–response (log-transformed) curves from the test samples and reference antigen are parallel (common slopes). Before any result can be generated by this method, a statistical test for parallelism must be performed (usually an F-test).

Manufacturers often supply vaccines worldwide and thus need to comply with different specifications or requirements. For instance, in order to supply the US market, the potency of all influenza vaccines needs to be calculated using PLA, although slope-ratio is mostly used elsewhere. Moreover, although formulations are the same for all markets for a given product, release specifications can vary. For instance, the US market requires the product to be released according to the average HA content and standard deviation of n gels. The lower specification limit for the average HA concentration is equivalent to 90 % of the label claim with a standard deviation corresponding to 12.3 % with $n = 3$ gels or 10.0 % for $n = 6$ gels. Alternatively, the European Pharmacopeia states that the lower 95 % confidence limits of the potency assay should indicate a content of at least 80 % of the label claim. Thus, a single product could have different estimated HA contents or be in or out of specifications based on the targeted market.

4.5.2 Assay Validation

SRID is a quantitative assay that requires validation or partial validation for every new strain tested. SRID falls into the category of a quantitative assay for the active moiety in a sample of drug substance or drug product. The SRID assay is validated for both drug substance samples as well as drug product. As a quantitative assay, the applicable ICH validation parameters include precision, accuracy, linearity, range, and specificity. Additionally, limit of quantitation is an important parameter that should be demonstrated during a validation study. The SRID can be sensitive to small changes in analytical conditions. Thus, a comprehensive robustness evaluation should be undertaken during development and validation.

Challenging specificity of the assay is fairly straightforward for both monovalent bulk material as well as drug product (seasonal). Specificity is the ability to assess unequivocally the analyte in the presence of components expected to be present. For determination of specificity (monovalent bulk), the bulk sample will be tested using homologous antibodies and antibodies specific for another strain. Zones of precipitation should be observed when the monobulk is tested with homologous antibodies and there should be no precipitation zone with heterologous antibodies. A similar approach can be taken to test trivalent or quadrivalent materials. A test preparation can be generated that includes two out of the three or four strains of interest. Different preparations would be made for each combination of strains. Zones of precipitation should be observed when testing with homologous antibodies and precipitation zones should not be observed when testing with heterologous antibodies. Accuracy is inextricably linked to method specificity for SRID as there is not a viable heterologous assay available to demonstrate accuracy. Assessment of accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted as the true value or an accepted reference value and the value found. With SRID, the reference material has an accepted true value and is used in the SRID assay to calculate a relative potency value for an unknown sample. One approach to demonstrating accuracy of the SRID assay is to perform spike-recovery studies across the range of the assay. Spiking a known amount of reference antigen into the test sample should result in an acceptable calculated recovery.

Linearity and limit of quantitation (LOQ) are critical parameters and must be assessed during validation. Evaluation of linearity is designed to determine if the SRID assay produces test results which are directly proportional to the concentration of analyte in the sample. The linear relationship must be evaluated across the range of the assay and can be done by dilution of the test sample to five concentrations that span the range of the assay. Visual inspection of the plot of theoretical potency values verse the observed potency value can be useful. Calculating the correlation coefficient (R), y-intercept, slope of the regression line, and residual sum of squares can also be beneficial in assess linearity. An R value of ≥ 0.95 should be expected. The LOQ can be, depending upon the strain and other factors, a challenge for SRID. LOQ evaluation should determine the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. This is

generally in the range of 7–10 μg/mL for the SRID assay. The LOQ for SRID can be determined based upon the standard deviation of the response and the slope. However, any calculation of LOQ should be confirmed empirically during the validation. The LOQ should be within the linear range, have acceptable spike recoveries at that concentration, and have suitable precision. The range of the assay is determined much like any other quantitative assay. The range of SRID is the interval between the upper and lower concentrations where it is demonstrated that the assay provides results that the linear, accurate, and precise.

In 1981, variability of the SRID assay was evaluated across 25 laboratories and 14 countries (Wood et al. 1981). Although this study focused on reproducibility, it highlights the role assay variability plays in having a quantitative potency assay that is suitable for its intended use. During validation, determination of assay precision is an integral part to demonstrating a quantitative assay is fit for purpose. Variability of the SRID assay is dependent upon numerous factors. One major factor can be the virus strain used as well as production system. Sharpness of the precipitation ring is dependent upon strain type and also the type of production system (i.e., any production system other than the traditional egg-based system). "Fuzzy" precipitation rings makes it difficult to measure the zone and cause an increase in assay variability. Other factors influencing the precision of the method include plate-to-plate variability, analyst variability, and variability due to different reagent sets. All of these factors must be assessed during the validation of repeatability and intermediate precision. Regardless of experimental design, the precision studies must show the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Due to the nature of the SRID assay, acceptance criteria for precision can be as high as a RSD of 20 %.

Robustness of any analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. This is particularly important for potency assays such as SRID. There are many steps in the SRID assay that could be tested to ensure robustness of the assay because many of the critical steps are performed manually. These include pouring of the gel, incubation times and temperatures, drying time for gels, stability of standards and reagents, and plate storage time. The outcome of these robustness studies should be an understanding of the factors which can cause assay variability. System suitability parameters can be designed around this knowledge of factors influencing assay robustness.

4.5.3 Reference Reagents Quality and Availability

An important rate-limiting step in the pandemic and seasonal vaccine supply process is the availability of the reference reagents for testing and release of the vaccine. Currently, approximately 3 months are required from the moment a reassortant/strain to be included in a vaccine is identified and the time calibrated reference reagents are

available to manufacturers. Six major steps usually take place during these 3 months: (1) amplification of the reassortant in sufficient quantities for antiserum and antigen production, (2) purification of HA by bromelain, (3) sheep immunization and bleeding, (4) antiserum characterization, (5) antigen calibration, and (6) reagents filling, packaging, and delivery. Of these six steps, two are critical from a manufacturing standpoint.

Antiserum characterization is a critical and rate-limiting step from a manufacturing perspective. This is especially true for the seasonal vaccines when three or four different strains are formulated together. Reference antiserum used must be specific to its homologous antigen. Cross-reactivity between strains must be kept at a minimum in order to demonstrate strain identity and to allow accurate quantitation of each strain.

Antigen calibration is also critical and rate-limiting from a manufacturing perspective. The calibration procedure is a cooperative action aimed at producing comparable and consistent reference antigens. First, the primary antigen, purified virus, is provided by one of the four reference laboratories. It is characterized for total protein content by Lowry, nitrogen content, or other recognized protein methods. The HA-to-protein ratio is then evaluated by densitometry on SDS-PAGE. With both data, HA content of the reference antigen can be calculated. The reference antigen can then be used to calibrate secondary antigens supplied by other reference laboratories by SRID (World Health Organization 2012a). Because each manufacturer evaluates the amount of antigen produced by comparison with a reference antigen, the declared potency value has an enormous impact on the calculated yield. An overestimated reference value leads to a lower global manufacturing capacity and vice versa. Moreover, as one reagent lot is not sufficient to fulfill all the manufacturers demand, multiple reagent lots are used throughout a manufacturing campaign. Manufacturing consistency can thus only be achieved if reagents are equally consistent.

Although many efforts are made to standardize reagents across lots and suppliers, differences have been observed between the declared potency values of these reagents and the values measured by the manufacturers. These in turn have a huge impact on the estimated potency of the vaccines. Some countries require exclusive usage of one SRID reagent supplier for potency testing unless specifically authorized (for instance USA and CBER reagents). Thus, a manufacturer producing vaccines for more than one market could potentially test the same product with different sets of reagents and obtain significantly different potency results, creating a discrepancy.

One potentially interesting solution to the issues mentioned at above could be the use of universal antisera for the quantitative evaluation of HA content in all subtypes of influenza vaccines. Such an antiserum would require to target a conserved portion of the hemagglutinin protein. A small region common to A and B strains has been identified and antisera generated against that epitope (Chun et al. 2008; Gravel et al. 2010). Interesting results were obtained by alternative methods like ELISA, but no application in SRID has yet been reported.

4.5.4 Challenge of Using these Reagents with Novel Production Systems

The SRID assay is used to evaluate HA content and to monitor potency and stability of the vaccine over time. Specific reference reagents are produced following the annual recommendation for vaccine composition by the WHO. The reference antigen reagents used in the SRID assay come from viruses grown in embryonic eggs or cell culture and against which a reference antiserum is produced in sheep.

The process by which these reagents are produced limits their applicability for vaccines made other manufacturing technologies. First, the production of influenza viruses in a given platform could induce some mutations on the surface proteins of the influenza viruses against which the reference antiserum is developed. In fact, influenza viruses have the ability to mutate their surface proteins to adapt to host cells. Such mutation will occur in any production system supporting the growth of influenza viruses. For example, influenza viruses isolated from humans have a preference for alpha 2–6 sialic acid receptors whereas avian influenza viruses would rather bind alpha 2–3 sialic acid receptors (Gambaryan et al. 1997). In general, a human strain grown in eggs will produce low virus yields. These yields can be improved by several passage in embryonic eggs or by using a reassortment process but unfortunately this process could lead to amino acid mutations in the surface proteins (HA and NA), especially in the receptor-binding domain of the HA molecule or in the glycosylation sites found at the tip of the molecule (Robertson et al. 1985, 1991; Gambaryan et al. 1999).

Plant-based or insect cell culture manufacturing technologies use the genetic information coding for the influenza proteins of a given strain to initiate vaccine production. Therefore, most vaccine developers using these novel production systems will use the genetic information of strains recommended by WHO that are isolated from humans rather than reassortant strains adapted for growth in eggs. It is believed that human sequences are more consistent to influenza viruses circulating in humans than egg-grown reassortant strains and are preferred sequences when developing an influenza vaccine for humans. Also, the recombinant technologies are not based on the growth of influenza viruses. Therefore, the mutation process which takes place when an influenza virus adapts to a given cell culture system or embryonic egg does not occur with recombinant technologies.

One could think that reference SRID reagents that are mostly derived from egg-adapted virus antigens and associated antiserum will show a bias when evaluating the HA content of an influenza vaccine made by an novel production system, especially if this system supports the production of influenza antigens based on wild-type genetic sequences. In fact, SRID reagents are developed for viruses that do not match the genetic sequence used by most recombinant technologies.

Typically, 3–9 N-linked glycans are attached to the HA protein backbone (Schulze 1997). The number and type of attached oligosaccharides strongly depend on the virus subtype (Gething et al. 1980; Verhoeyen et al. 1980; Hiti et al. 1981) and strain (Schwarz and Klenk 1981; Wagner et al. 2000). Further, it is known that

N-linked oligosaccharides attached to the stalk region are highly conserved, whereas those at the tip of the molecule vary considerably in structure and number among different influenza viruses (Wagner et al. 2000). Also, N-glycosylation of HA is dependent on the expression system and could lead to different affinities for the reference SRID antibody reagent.

4.5.4.1 Proposed Solutions

Develop SRID reagents specific to a given strain/manufacturing technology.

One company developing Influenza Virus-Like Particles produced in insect cells used that strategy for the release of their trivalent influenza vaccine (Pincus et al. 2010). The authors reported that the egg-derived or the baculovirus-derived SRID reagents were in correlation for the quantification of H1N1 and B strains but not for the H3N2 and they concluded that the baculovirus-derived reagents evaluated that strain with more accuracy.

Evaluate the HA content using a total protein assay and use SRID assay to evaluate relative content and to monitor stability.

This solution can be used for HA-based vaccine and is not suitable for vaccine containing several influenza proteins. One example of HA-based vaccine is Medicago's VLPs formed of the hemagglutinin protein of the influenza virus transiently expressed in the plant cells and that assemble into a "carrier" non-infectious virus-like structure, with a plant lipid bilayer and protruding HA spikes. Plant-made VLP vaccines are over 90 % pure of HA protein as evaluated by Coomassie-stained gels and band identification by Western blot analysis and mass spectrometry. Plant-made VLPs have been tested in three clinical trials (Landry et al. 2010). The discrepancy between the SRID reading and the total protein assay varies depending on selected influenza strains (data not shown). For the pandemic H1 VLP vaccine evaluated during a Phase 1 clinical trial in healthy adults in the USA (manuscript in preparation), it was agreed with the regulators to base HA dosages on the total protein assay and to use SRID for relative quantitation and stability monitoring. Medicago used the HA sequence from the A/California/7/09 H1N1 strain isolated from one patient. That HA sequence shows four amino acids difference in the HA molecule compared to the X-179A H1N1 reassortant strain used for vaccine production in embryonic eggs and for the production of SRID reagents. For that strain, the standard SRID test overevaluated the HA content by 30 %.

Even though the total protein assay was used to standardize vaccine dosages, the SRID assay was used during stability monitoring and to show variation of potency overtime.

Develop reference reagents specific for a manufacturing technology.

That is the optimal solution that was adopted for cell-based influenza vaccines. In the United States, CBER is developing reference reagents for virus grown in both embryonic eggs and MDCK cell line.

4.6 New Influenza Vaccine Formulations

4.6.1 Quadrivalent Seasonal Formulation

B strains from both lineages, Yamagata and Victoria, often co-circulate in the population (Belshe 2010). Thus, there is always a risk that the strain included in the vaccine formulation does not protect against the most prevalent strain during the flu season. In fact, the match between the vaccine and prevalent B strains only occurred in 5 out of 10 flu seasons between 2001 and 2010 (Belshe 2010). To fill this gap, quadrivalent formulations including B strains from both lineages are now commercially available (quadrivalent influenza vaccine or QIV).

From an analytical standpoint, this new formulation represents a challenge. Although both lineages are antigenically distinguishable using HAI or microneutralization, once treated and analyzed by immunodiffusion with typical reference antisera, cross-reactivity between strains was observed (Gupta et al. 2011). In turn, this leads to two different issues: (1) identity testing is no longer feasible for the B strains, because an antigen is recognized by the heterologous antiserum and, (2) overestimation of the potency.

Concerning the identity testing issue, by comparing the results obtained with CBER reagents to TGA and NIBSC reagents, it was demonstrated that CBER antisera against B strain lacked specificity for the analysis of QIV samples (in 2011). It was hypothesized that this could be due to differences in the sheep immunization protocols between CBER and other reference laboratories. Supporting this hypothesis is the fact that CBER later produced more specific antisera using a modified immunization protocol. This modified protocol resulted in antisera which could be used for identity testing (Gupta et al. 2011).

The potency overestimation issue is more complex. Even when no evident cross-reactivity is observed with the identity test using antisera from all reagent suppliers, variable degrees of overestimation from null to high can be observed depending on the reagent/supplier used and the strain analyzed. As shown in Fig. 4.3, it is hypothesized that even with specific polyclonal antisera a small subset of antibodies react against common epitopes in both B strains leading to increase precipitation rings. To alleviate this issue, it was recommended to prepare a mixture of reference antigens in the standard curve for both B strains (Gupta et al. 2011). As reference antigens behave the same way as samples, thus sharing the same epitopes, no overestimation is expected.

4.6.2 Formulations Containing Adjuvants

Formulations containing adjuvants have been designed to enhance the immune reaction to vaccines. Depending on the type of adjuvant, the impact on SRID is more or less observed.

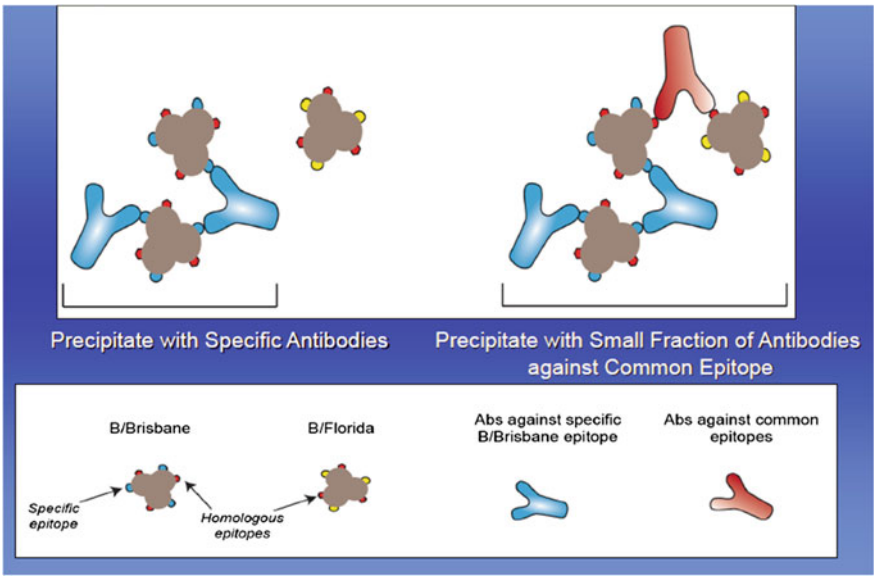


Fig. 4.3 Hypothesis for the overestimation of the HA content in quadrivalent vaccine formulations (excerpt from Gupta et al. 2011)

Aluminum precipitation or adsorption has been one of the first and the most widely used form of adjuvantation for vaccines. However, aluminum-adsorbed influenza vaccines have been shown to be difficult to assay directly by SRID due to the tightly bound aluminum masking the epitopes recognized by the antiserum. To measure potency in the final formulation, analytical strategies like desorption have been used (Sizer et al. 2008). However, in most cases, a theoretical evaluation of the HA content is made based on the HA content before the addition of the adjuvant.

Other types of adjuvants like oil-in-water emulsions have been used in the last decades. Although limited literature describes specifically the modifications to the SRID assay, it is increasingly evident that the addition of these adjuvants affects the method performance (Baras and Jacob 2010).

Finally, the advantage of adjuvantation is often to reduce the amount of antigen needed for each dose. Indeed, adjuvanted vaccine doses have been shown to be effective in doses as low as 3.75 μg/ml (Hehme et al. 2002). On the other hand, this generates an additional challenge from an analytical point of view, because these concentrations are well below the SRID limit of quantification is generally around 5–10 μg/ml. Thus, even when adjuvants do not interfere, formulations containing such low HA content require a modified SRID assay or an alternative potency assay.

4.6.3 Alternative Potency Assays

Several attempts have been made to replace the SRID assay especially in pandemic scenarios during which the time required to produce reference reagents delay the availability of the vaccines on the market. So far, all of the proposed assays can be used to evaluate HA content and concentration but none of them can really ensure the potency of influenza vaccines. The main reason for this is that no single method has achieved all the following characteristics:

- Accuracy and precision greater or equal to the current SRID
- Dynamic range of measurement greater or equal to the current SRID
- Potency measured should correlate with current SRID results
- Capable of measuring potency of vaccine strain subtypes in a trivalent (multivalent) vaccine
- Stability indicating—capable of quantifying subpotent vaccines
- Transferable and practical

A recent workshop on Alternative Potency Assays for Influenza Vaccines was held in London in July 2013. Many alternative assays were presented by flu vaccine manufacturers, regulatory agencies, and suppliers of analytical technologies. These included immunological assays like the ELISA, based on conserved antigen sequences (Gravel et al. 2010; Li et al. 2010) or on glycans for egg-derived influenza vaccines (Legastelois et al. 2011; Hashem et al. 2013), immunological assays using surface plasmon resonance (Nilsson et al. 2010), reverse-phase and size-exclusion HPLC methods (Kapteyn et al. 2006, 2009; Lorbetskie et al. 2011), and recently isotope-dilution mass spectrometry (Williams et al. 2008) and label-free MS-based methods (Creskey et al. 2012). Preliminary results from a novel technology called Titer on Chip™ were also presented (InDevR 2013). This multiplexed assay, based on microarray technology, relies on monoclonal antibodies that recognize conserved sequences in the different subtypes of influenza hemagglutinin (e.g., H1, H3, H5, B, etc.). Provided that this technology works robustly with vaccines from different manufacturers and formulations, it could potentially achieve many of the desired characteristics for an alternative potency assay (described above).

The main expected advantage of these alternative methods is their increased sensitivity over traditional SRID assays and their better accuracy and precision over a large range of concentration. This is proven particularly useful with the development of adjuvanted influenza vaccines that are administered at doses below the current 15 μg per strain per dose.

Accuracy of SRID for the evaluation of HA content

Interestingly, MS-based assays, RP-HPLC techniques, and immunological assays revealed important limitations of the SRID assay to accurately quantify the HA content even in egg-derived vaccines. The HA content of the reference antigen SRID reagent is evaluated based on a total protein assay and by performing relative quantification of all viral proteins by SDS-PAGE and gel densitometry measurements

(described above). The reliability of this technique greatly depends on the protein content, the ability of all proteins to migrate according to their molecular weight and to be visible on the gel and the absence of protein degradation aggregation. Any inaccuracy in the HA concentration of the reference SRID antigen reagent will propagate as inaccuracies in the evaluation of HA content of influenza vaccines released based on that SRID reagent. One paper describes the difference in HA content values between the SRID assay and one LC-MS technique (Creskey et al. 2012). The authors reported that the HA content of the reference SRID antigens evaluated by SDS-PAGE and gel densitometry represents 66 to more than 200 % of the target value according to the LC-MS assay. An analysis of seven pandemic H1N1 vaccines revealed that those vaccines were at the target concentration of 15 μg per dose according to the SRID assay but between 33 and 67 % of the target value according to the LC-MS technique. The same technique also suggests that the H1N1 component of seasonal trivalent vaccines is in general better evaluated by SRID than the H3N2 and B components, the latter being largely overestimated by SRID assays. The LC-MS assay showed good linearity and accuracy and the conclusions are in accordance with other techniques such as isotope-dilution mass spectrometry (Williams et al. 2008) or RP-HPLC (Lorbetskie et al. 2011), and immunological assays (Nilsson et al. 2010).

The SRID assay is a mean by which vaccine doses can be standardized according to their immunological potential. The case of pandemic vaccines described above (Creskey et al. 2012) shows that all vaccines had a similar SRID value despite different total content in HA which can be caused by different alteration of the HA protein during the process of virus isolation and disruption.

4.7 Conclusion

Influenza vaccines are unique from a manufacturing and analytical perspective. For seasonal vaccines, as strains change yearly, a “new” vaccine is consequently formulated every year. This constitutes a manufacturing risk as the addition of each new strain to a formulation can potentially alter the physicochemical and immunogenic properties of the final product and/or its stability or safety profile. As timing for delivery of the vaccines to the patients is critical, especially during a pandemic outbreak, manufacturers put great efforts to go through all the manufacturing process steps, characterization, and release testing in the shortest amount of time. However, many bottlenecks in the lead time to market do not depend on the manufacturers.

In the last 10–20 years, the influenza vaccines landscape has tremendously evolved. Many formulations have been developed to increase vaccine efficacy. While egg-based manufacturing is the oldest and most common process for vaccine production, alternative processes aiming at decreasing processing times or circumventing dependence to eggs are now or soon to be available. Classical SRID is not always fit for purpose with these newer vaccine formulations. To overcome this, various analytical strategies and reagents are being developed, but still await the approval by the authorities.

Finally, even when the classical SRID is applicable, some issues still remain with the test. Indeed, one major bottleneck is the supply of reference reagents for the main potency assay SRID which relies on different interacting entities (WHO, ERL, manufacturers). In addition to supply issues, many weaknesses were shown over the years with the robustness/ruggedness of this bioassay. For all of these reasons, intensive development work is still being done on the analytical tools for what is an apparently "old" vaccine.

References

Abelin A, Colegate T, Gardner S, Hehme N, Palache A (2011) Lessons from pandemic influenza A(H1N1): the research-based vaccine industry's perspective. Vaccine 29:1135–1138

Alexander DJ (2000) A review of avian influenza in different bird species. Vet Microbiol 74:3–13

Baras BGJ, Jacob VAM (2010) Immunodiffusion assay for influenza virus. [US 2012/0178185 A1]. 9-23-2010. United States

Belshe RB (2010) The need for quadrivalent vaccine against seasonal influenza. Vaccine 28(Suppl 4):D45–D53

Bogs J, Veits J, Gohrbandt S, Hundt J, Stech O, Breithaupt A, Teifke JP, Mettenleiter TC, Stech J (2010) Highly pathogenic H5N1 influenza viruses carry virulence determinants beyond the polybasic hemagglutinin cleavage site. PLoS ONE 5:e11826

Chun S, Li C, Van DG, Wang J, Farnsworth A, Cui X, Rode H, Cyr TD, He R, Li X (2008) Universal antibodies and their applications to the quantitative determination of virtually all subtypes of the influenza A viral hemagglutinins. Vaccine 26:6068–6076

Creskey MC, Li C, Wang J, Girard M, Lorbetskie B, Gravel C, Farnsworth A, Li X, Smith DG, Cyr TD (2012) Simultaneous quantification of the viral antigens hemagglutinin and neuraminidase in influenza vaccines by LC-MSE. Vaccine 30:4762–4770

Dormitzer PR, Tsai TF, Del GG (2012) New technologies for influenza vaccines. Hum Vaccin Immunother 8:45–58

Doroshenko A, Halperin SA (2009) Trivalent MDCK cell culture-derived influenza vaccine Optaflu (Novartis vaccines). Expert Rev Vaccines 8:679–688

Engelhardt OG (2012) Many ways to make an influenza virus—review of influenza virus reverse genetics methods. Influenza and Other Respir Viruses. doi: 10.1111/j.1750-2659.2012.00392.x

Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, Garcia-Sastre A (1999) Rescue of influenza a virus from recombinant DNA. J Virol 73:9679–9682

Friede M, Palkonyay L, Alfonso C, Pervikov Y, Torelli G, Wood D, Kieny MP (2011) WHO initiative to increase global and equitable access to influenza vaccine in the event of a pandemic: supporting developing country production capacity through technology transfer. Vaccine 29(Suppl 1):A2–A7

Gambaryan AS, Robertson JS, Matrosovich MN (1999) Effects of egg-adaptation on the receptor-binding properties of human influenza A and B viruses. Virology 258:232–239

Gambaryan AS, Tuzikov AB, Piskarev VE, Yamnikova SS, Lvov DK, Robertson JS, Bovin NV, Matrosovich MN (1997) Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6'-sialyl (N-acetyllactosamine). Virology 232:345–350

Gething MJ, Bye J, Skehel J, Waterfield M (1980) Cloning and DNA sequence of double-stranded copies of haemagglutinin genes from H2 and H3 strains elucidates antigenic shift and drift in human influenza virus. Nature 287:301–306

Gravel C et al (2010) Qualitative and quantitative analyses of virtually all subtypes of influenza A and B viral neuraminidases using antibodies targeting the universally conserved sequences. Vaccine 28:5774–5784
Gupta RK, Semenova EA, McCormick WM (2011) Guidance on SRID method for the inactivated influenza virus vaccines. In: PhRMA Annual Meeting 12-15-2011
Hashem AM, Gravel C, Farnsworth A, Zou W, Lemieux M, Xu K, Li C, Wang J, Goneau MF, Merziotis M (2013) A novel synthetic receptor-Based immunoassay for influenza vaccine quantification. PLoS ONE 8:e55428
Hay AJ, Gregory V, Douglas AR, Lin YP (2001) The evolution of human influenza viruses. Philos Trans R Soc Lond B Biol Sci 356:1861–1870
Hehme N, Engelmann H, Kuenzel W, Neumeier E, Saenger R (2004) Immunogenicity of a monovalent, aluminum-adjuvanted influenza whole virus vaccine for pandemic use. Virus Res 103:163–171
Hehme N, Engelmann H, Kunzel W, Neumeier E, Sanger R (2002) Pandemic preparedness: lessons learnt from H2N2 to H9N2 candidate vaccines. Med Microbiol Immunol 191:203–208
Hiti AL, Davis AR, Nayak DP (1981) Complete sequence analysis shows that the hemagglutinins of the H0 and H2 subtypes of human influenza virus are closely related. Virology 111:113–124
InDevR (2013) Titer on chip. http://indevr.com/products/in-the-works/. 11-28-2013. Ref Type: Internet Communication
Kapteyn JC et al (2009) HPLC-based quantification of haemagglutinin in the production of egg- and MDCK cell-derived influenza virus seasonal and pandemic vaccines. Vaccine 27:1468–1477
Kapteyn JC et al (2006) Haemagglutinin quantification and identification of influenza A&B strains propagated in PER.C6 cells: a novel RP-HPLC method. Vaccine 24:3137–3144
Kieny MP et al (2006) A global pandemic influenza vaccine action plan. Vaccine 24:6367–6370
Kilbourne ED (1969) Future influenza vaccines and the use of genetic recombinants. Bull World Health Organ 41:643–645
Killian M (2008) Hemagglutination assay for the avian influenza virus. In: Avian influenza virus (ed). E.Spackman Humana Press, New York, pp 47–52
Kistner O, Barrett PN, Mundt W, Reiter M, Schober-Bendixen S, Dorner F (1998) Development of a mammalian cell (Vero) derived candidate influenza virus vaccine. Vaccine 16:960–968
Klimov A, Balish A, VeguillaV, Sun H, Schiffer J, Lu X, Katz JM, Hancock K (2012) Influenza virus titration, antigenic characterization, and serological methods for antibody detection. In: Influenza virus, Springer, Berlin, pp 25–51
Landry N, Ward BJ, Trepanier S, Montomoli E, Dargis M, Lapini G, Vezina LP (2010) Preclinical and clinical development of plant-made virus-like particle vaccine against avian H5N1 influenza. PLoS ONE 5:e15559
Legastelois I et al (2011) Avian glycan-specific IgM monoclonal antibodies for the detection and quantitation of type A and B haemagglutinins in egg-derived influenza vaccines. J Virol Methods 178:129–136
Li C, Jaentschke B, Song Y, Wang J, Cyr TD, Van DG, He R, Li X (2010) A simple slot blot for the detection of virtually all subtypes of the influenza A viral hemagglutinins using universal antibodies targeting the fusion peptide. Nat Protoc 5:14–19
Lorbetskie B, Wang J, Gravel C, Allen C, Walsh M, Rinfret A, Li X, Girard M (2011) Optimization and qualification of a quantitative reversed-phase HPLC method for hemagglutinin in influenza preparations and its comparative evaluation with biochemical assays. Vaccine 29:3377–3389
Minor PD (2010) Vaccines against seasonal and pandemic influenza and the implications of changes in substrates for virus production. Clin Infect Dis 50:560–565
Neumann G et al (1999) Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci USA 96:9345–9350
Nichol KL (2008) Efficacy and effectiveness of influenza vaccination. Vaccine 26:D17–D22
Nichol KL, Treanor JJ (2006) Vaccines for seasonal and pandemic influenza. J Infect Dis 194 (Suppl 2):S111–S118

Nicolson C, Major D, Wood JM, Robertson JS (2005) Generation of influenza vaccine viruses on Vero cells by reverse genetics: an H5N1 candidate vaccine strain produced under a quality system. Vaccine 23:2943–2952

Nilsson CE, Abbas S, Bennemo M, Larsson A, Hamalainen MD, Frostell-Karlsson A (2010) A novel assay for influenza virus quantification using surface plasmon resonance. Vaccine 28:759–766

Osterholm MT, Kelley NS, Sommer A, Belongia EA (2012) Efficacy and effectiveness of influenza vaccines: a systematic review and meta-analysis. Lancet Infect Dis 12:36–44

Pincus S, Boddapati S, Li J, Sadowski T (2010) Release and stability testing programs for a novel virus-like particle vaccine

Robertson JS, Cook P, Nicolson C, Newman R, Wood JM (1994) Mixed populations in influenza virus vaccine strains. Vaccine 12:1317–1322

Robertson JS, Naeve CW, Webster RG, Bootman JS, Newman R, Schild GC (1985) Alterations in the hemagglutinin associated with adaptation of influenza B virus to growth in eggs. Virology 143:166–174

Robertson JS, Nicolson C, Bootman JS, Major D, Robertson EW, Wood JM (1991) Sequence analysis of the haemagglutinin (HA) of influenza A (H1N1) viruses present in clinical material and comparison with the HA of laboratory-derived virus. J Gen Virol 72(Pt 11):2671–2677

Robertson JS, Nicolson C, Major D, Robertson EW, Wood JM (1993) The role of amniotic passage in the egg-adaptation of human influenza virus is revealed by haemagglutinin sequence analyses. J Gen Virol 74(Pt 10):2047–2051

Rodda SJ, Gallichio HA, Hampson AW (1981) The single radial immunodiffusion assay highlights small antigenic differences among influenza virus hemagglutinins. J Clin Microbiol 14:479–482

Schulze IT (1997) Effects of glycosylation on the properties and functions of influenza virus hemagglutinin. J Infect Dis 176(Suppl 1):S24–S28

Schwarz RT, Klenk HD (1981) Carbohydrates of influenza virus. IV. Strain-dependent variations. Virology 113:584–593

Sizer PJ, King-Haughey J, Simpkin D, Williams R (2008) Assays for adsorbed influenza vaccines. 12/810,307[US 2011/0045457 A1]. 12-24-2008. United States. Ref Type: Patent

Stephenson I, Nicholson KG, Gluck R, Mischler R, Newman RW, Palache AM, Verlander NQ, Warburton F, Wood JM, Zambon MC (2003) Safety and antigenicity of whole virus and subunit influenza A/Hong Kong/1073/99 (H9N2) vaccine in healthy adults: phase I randomised trial. Lancet 362:1959–1966

Suarez DL (2010) Avian influenza: our current understanding. Anim Health Res Rev 11:19–33

Tong S et al (2012) A distinct lineage of influenza A virus from bats. Proc Natl Acad Sci USA 109:4269–4274

Treanor JJ, Wilkinson BE, Masseoud F, Hu-Primmer J, Battaglia R, O'Brien D, Wolff M, Rabinovich G, Blackwelder W, Katz JM (2001) Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans. Vaccine 19:1732–1737

van Kessel G, Geels MJ, de Weerd S, Buijs LJ, de Bruijni MA, Glansbeek HL, van den Bosch JF, Heldens JG, van den Heuvel ER (2012) Development and qualification of the parallel line model for the estimation of human influenza haemagglutinin content using the single radial immunodiffusion assay. Vaccine 30:201–209

Veits J et al (2012) Avian influenza virus hemagglutinins H2, H4, H8, and H14 support a highly pathogenic phenotype. Proc Natl Acad Sci USA 109:2579–2584

Verhoeyen M, Fang R, Jou WM, Devos R, Huylebroeck D, Saman E, Fiers W (1980) Antigenic drift between the haemagglutinin of the Hong Kong influenza strains A/Aichi/2/68 and A/Victoria/3/75. Nature 286:771–776

Wagner R, Wolff T, Herwig A, Pleschka S, Klenk HD (2000) Interdependence of hemagglutinin glycosylation and neuraminidase as regulators of influenza virus growth: a study by reverse genetics. J Virol 74:6316–6323

Wareing MD, Tannock GA (2001) Live attenuated vaccines against influenza; an historical review. Vaccine 19:3320–3330

Webby RJ, Perez DR, Coleman JS, Guan Y, Knight JH, Govorkova EA, Clain-Moss LR, Peiris JS, Rehg JE, Tuomanen EI (2004) Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. Lancet 363:1099–1103

Williams MS (1993) Single-radial-immunodiffusion as an in vitro potency assay for human inactivated viral vaccines. Vet Microbiol 37:253–262

Williams TL, Luna L, Guo Z, Cox NJ, Pirkle JL, Donis RO, Barr JR (2008) Quantification of influenza virus hemagglutinins in complex mixtures using isotope dilution tandem mass spectrometry. Vaccine 26:2510–2520

Wood JM, Schild GC, Newman RW, Seagroatt V (1977) An improved single-radial-immunodiffusion technique for the assay of influenza haemagglutinin antigen: application for potency determinations of inactivated whole virus and subunit vaccines. J Biol Stand 5:237–247

Wood JM, Seagroatt V, Schild GC, Mayner RE, Ennis FA (1981) International collaborative study of single-radial-diffusion and immunoelectrophoresis techniques for the assay of haemagglutinin antigen of influenza virus. J Biol Stand 9:317–330

World Health Organization (2006) Global pandemic influenza action plan to increase vaccine supply. In: Immunization, vaccines, and biologicals, epidemic and pandemic alert and response. WHO, Geneva, Switzerland

World Health Organization (2009) Influenza Fact sheet 2011. http://www.who.int/mediacentre/factsheets/fs211/en. Ref Type: Generic

World Health Organization (2012a) Geneva, 17 to 21 October 2011 Generic protocol for the calibration of seasonal/pandemic influenza antigen working reagents by WHO Essential Regulatory Laboratories. Ref Type: Report

World Health Organization (2012b) WHO global influenza surveillance network. Manual for the laboratory diagnosis and virological surveillance of influenza. Ref Type: Generic

Young SA (2000) Clinical virology manual. Am Soc Microbiol

Zhang W et al (2011) Increase in viral yield in eggs and MDCK cells of reassortant H5N1 vaccine candidate viruses caused by insertion of 38 amino acids into the NA stalk. Vaccine 29:8032–8041

Chapter 5
Live-Attenuated and Inactivated Whole-Cell Bacterial Vaccines

Anita H.J. van den Biggelaar and Jan T. Poolman

5.1 Introduction

During the mid-to-late nineteenth century, growing interest in the concept of pathogen attenuation to allow its safe administration to protect a susceptible subject against disease or infection, led to the development by Louis Pasteur of vaccines against chicken cholera, animal anthrax, and the first live-attenuated human vaccine, which was for rabies. At the same time, the notion that killed bacteria were capable of immunizing was being explored in the United States, with the development of a killed hog salmonella vaccine, followed by the first human killed vaccines for typhoid and cholera. Thus, the group of live-attenuated and killed whole-cell bacterial vaccines includes some of the oldest human vaccines still in use today. Two of them, namely Bacille Calmette-Guérin (BCG) and whole-cell pertussis vaccines, have been administered to billions of individuals over many decades of use, and continue to be widely used in universal infant vaccination programs.

For the diseases targeted by live-attenuated and killed whole-cell bacterial vaccines, the immune mechanisms of protection remain incompletely understood and serological correlates of protection have not been identified, apart from whole cell pertussis vaccines for which bacterial agglutination has been proposed as a link to protection. To some extent this has hindered the development of more effective vaccines.

The production of live-attenuated and killed bacterial vaccines has been troubled historically by issues of quality and poor efficacy. Incomplete attenuation is associated with the risk of acute infection and disease by the vaccine strain. Production

A.H.J. van den Biggelaar · J.T. Poolman (✉)
Bacterial Vaccines—Discovery and Early Development, Crucell, Archimedesweg 4-6, 2333CN Leiden, The Netherlands
e-mail: JPoolman@its.jnj.com

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6_5

of inactivated vaccines of inconsistent potency and quality has led to outbreaks of disease in countries where vaccine of low potency have been used. Despite these limitations, live-attenuated and killed whole-cell bacterial vaccines will continue to be used until new vaccines that provide higher levels of protection become available.

5.2 Bacille Calmette-Guérin Vaccine

5.2.1 Tuberculosis: Overview of Bacteriology and Pathology

Mycobacterium tuberculosis remains common in the twenty-first century, infecting not less than one-third of the global population (Donoghue et al. 2010; Robbins et al. 2009; World Health Organization 2004) and being responsible for an estimated 1.2–1.5 million tuberculosis deaths in 2010 (Lozano et al. 2012). *M. tuberculosis* is an aerobic rod with a complex cell wall containing high levels of mycolic acid: the unique characteristics of the cell wall mean that mycobacteria fail to decolourise in response to routine Gram staining, giving them their "acid-fast" characteristic. *M. tuberculosis* does not produce toxins but causes disease by living intracellularly in the host, mostly macrophages and granulocytes.

Clinical tuberculosis may take many forms that are influenced by the age and immune status of the patient, and include latent and active phases that together make detection and diagnosis challenging. Primary infection usually occurs in the lung apices after aerosol intake and develops over several weeks into a primary caseating nodule with systemic dissemination and granuloma formation. A relatively small number of individuals, usually children, develop tuberculous meningitis or miliary (disseminated) tuberculosis soon after primary infection, which are rapidly evolving life-threatening conditions. In most individuals, the primary complexes and foci of disseminated bacteria are walled off by the host immune system by the formation of granulomas, a process taking several weeks. This process is associated with the development of delayed cutaneous hypersensitivity. Once concealed, bacilli may lie dormant intracellulary within the host for years or decades, and the vast majority of *M. tuberculosis* infected persons (up to 95 % of infected adults) remain asymptomatic for life (World Health Organization 2004). Reactivation may occur at any time, but is most frequent during adolescence and adulthood, often following a period of immune-suppression, and usually involves reactivation in the lungs but potentially in any organ. Secondary or reactivated pulmonary tuberculosis causing chronic cough is highly contagious and is the main transmissible form of the disease.

Of the estimated 8.7 million new cases of active tuberculosis in 2011, the largest proportions occurred in Asia (59 %; mainly India and China) and Africa (26 %), compared to 3 % in the Americas (World Health Organization 2012a). Currently, the highest incidence of new cases per population is in South Africa, where there

were 993 new cases per 100,000 individuals in 2011. Immune compromised individuals are at greatest risk of reactivation and disease progression. Thirteen percent of new tuberculosis cases in 2011 were in individuals infected with HIV, most of whom (80 %) resided in Africa (World Health Organization 2012a). People living with HIV and infected with tuberculosis are 21–34 times more likely to develop active tuberculosis disease than people without HIV (World Health Organization 2011). Around one-third of annual deaths due to tuberculosis occur in individuals who are HIV positive (Lozano et al. 2012; World Health Organization 2012a).

The high rates of clinical tuberculosis among HIV-infected persons and persons receiving cytotoxic therapy point to a critical role of an intact adaptive immune system in containing the disease. The most important cellular player may be the macrophage, which has two contradictory roles in tuberculosis. On one hand, Th1-activated macrophages are capable of controlling the proliferation of *M. tuberculosis*, possibly through production of nitric oxide (Silva Miranda et al. 2012). On the other hand, uninfected Th2-induced macrophages produce anti-inflammatory cytokines that result in the development of an "immunosuppressive niche", allowing long-term survival of viable bacilli in infected macrophages (Qualls et al. 2010). Granulomas (nodules containing immune cells such as lymphocytes, neutrophils, eosinophils, macrophages, fibroblasts, and collagen that are formed when the immune system tries to bar the mycobacteria that it cannot eliminate) are present in persons with intact immunity but absent or poorly formed in persons with poor immune responses, supporting the notion that they are critical for limiting bacterial growth.

Disease control has been obstructed by the remarkable ability of the *M. tuberculosis* bacillus to persist for decades as latent infection in one-third of the global population, with the potential for reactivation at any time. Disease control is also severely hampered by the indolent nature of the disease, which often leads to late diagnosis allowing exposure of dozens or even hundreds of individuals to the index case. Antituberculosis drugs have been used for decades, but resistance to these medicines is growing. In recent years, multidrug resistant strains of *M. tuberculosis* have emerged, accounting for around 5 % of all tuberculosis cases globally (World Health Organization 2012a). Development of new antituberculosis drugs is an urgent need: an important step forward was the approval by the United States Food and Drug Administration (FDA) of *Bedaquiline* in 2012, the first medicine with a new mode of action in the treatment of TB in more than 40 years and the first and only one specifically indicated for multidrug resistant tuberculosis. Elimination of mycobacteria and cure requires unusually long treatment courses (up to 6 months) as compared to other bacterial infectious diseases. Methods that shorten treatment length are likely to improve compliance, successful bacterial clearance, and thus transmission.

5.2.2 Vaccines Licensed and in Use

5.2.2.1 Live-Attenuated Vaccines

Bacillus Calmette-Guérin is a live-attenuated strain of *Mycobacterium bovis* originally named after the two French scientists, Calmette and Guérin, who first isolated the BCG strain early in the twentieth century from a cow with tuberculous mastitis. The strain was subcultured over a period of 13 years to achieve attenuation. BCG was first used as a vaccine in humans in 1921 and was included as a pediatric vaccine in the World Health Organization Expanded Programme on Immunisation in 1974. In countries of high tuberculosis endemicity, BCG is typically administered soon after birth, but can be given at any age; in low endemic countries it is used to vaccinate high-risk groups (World Health Organization 2004).

Despite the length of experience, the mechanism of action by which BCG provides protection against *M. tuberculosis* disease remains incompletely understood. Production of interferon-gamma (IFN-γ) and interleukin-2 (IL-2) by $CD4^+$ T-helper 1 cells is thought to be critical (Chackerian et al. 2001; Cooper et al. 1993); however, the immunological interactions are complex with likely important roles for $CD8^+$ cells and many other effectors of the immune system (Ottenhoff and Kaufmann 2012; Ritz et al. 2008). Individuals with genetic defects in IFN-γ and IL-12 pathways are at higher risk of severe disease due to *M. tuberculosis* and non-virulent mycobacteria including BCG (Lee et al. 2011).

BCG is believed to be a strong enhancer of the neonatal innate immune system (Marchant and Goldman 2005; Watkins et al. 2008), inducing maturation of pro-inflammatory innate immune responses including the production of IL-12 that regulates the differentiation of naive T-helper cells into IFN-γ producing type 1 cells (Vekemans et al. 2001). A recent study demonstrated that vaccination with BCG induces two types of immune responses: classic, specific response involving antigen-specific T cells and memory, and "trained" innate immunity based on functional epigenetic reprogramming of mononuclear phagocytes involving NOD2-mediated epigenetic changes at the level of histone methylation (H3K4me3) (Kleinnijenhuis et al. 2012). This nonspecific maturational effect possibly explains the success of BCG as a neonatal vaccine (Burl et al. 2010).

In infected humans, BCG-activated T-cell responses are thought to contain the infection to the lungs, inhibiting haematogenous spread of organisms to peripheral tissues and the meninges. Thus, the major impact of BCG is the prevention of fulminant miliary tuberculosis and tuberculous meningitis in children, with efficacy against these endpoints estimated between 75 and 87 % (Trunz et al. 2006; Walker et al. 2006). Unlike many other vaccines, BCG does not prevent infection, and pulmonary *M. tuberculosis* infection appears to occur in individuals regardless of BCG vaccination status (Sutherland and Lindgren 1979). BCG does not prevent reactivation disease in adults and the duration of protection is not known.

5.2.3 Vaccines and Manufacturers

Current BCG vaccine strains are all derived from the original strain developed by Calmette and Guérin, and are stored by the World Health Organization as seed lots of lyophilised bacilli. Most of the currently available vaccines contain one of four strains, or their derivatives: Pasteur 1173 P2, Danish 1331, Glaxo 1077, or Tokyo 172. Currently used strains show genetic diversity, as well as variability in immunogenicity and their ability to induce cutaneous hypersensitivity (Milstien and Gibson 1990). Some strains provide greater protection in animal models than others, but these strains have also been linked with higher rates of side effects such as lymphadenitis and osteitis (Alrabiaah et al. 2012; Milstien and Gibson 1990).

Categorizing relationships between strain type, efficacy, and reactogenicity is confounded because culture methods have important effects on the biochemical characteristics of strains, which changes their efficacy in animal models (Venkataswamy et al. 2012). The evaluation of vaccine efficacy in humans is challenging because of the indolent nature of the main outcome: tuberculosis disease. Early studies that examined vaccine efficacy used passive surveillance methods without rigorous control of case definitions, and lacked methods to accurately characterize the strains under study. Other confounding factors such as age, disease stage, immunocompetence of the population studied, BCG administration route, prevalence of helminth infections, and prevalence of exposure to environmental mycobacteria preclude direct comparison of studies (Fine 1995). Nevertheless, a recent review concluded that available evidence supports the existence of differences between BCG strains in terms of immunogenicity and protective efficacy in humans, but the authors concluded that the data were insufficient to allow identification of a preferred strain (Ritz et al. 2008).

Worldwide, there are dozens of manufacturers producing BCG vaccines. Vaccines differ not only in strain type, but in chemical composition and the concentration of bacilli per dose. The viability of individual vaccines (that is, the proportion of dead versus live bacilli) also influences their clinical characteristics, with the total bacteria mass proportional to the degree of local reactogenicity, and the total number of viable bacteria proportional to the tuberculin sensitivity (Milstien and Gibson 1990). Vaccine quality was overseen by the World Health Organization until 1997. Since then, national regulatory agencies are responsible for quality control.

5.2.4 Limitations of BCG Vaccines

The role of antibodies in preventing and controlling *M. tuberculosis* infection is thought to be minimal (Schluger 2001), although some people argue that this is a neglected area of research. However, since activation of cell-mediated responses and development of these responses to a level where bacterial replication is

inhibited are slow, *M. tuberculosis* has many days to replicate unhindered after infection has occurred. Therefore, although efficacious in preventing severe forms of tuberculosis, BCG is poorly effective in preventing latent infection and subsequent reactivation. This is of critical epidemiological importance since the reactivated pulmonary form of tuberculosis is highly transmissible and to large extent responsible for ongoing circulation of tuberculosis globally.

In vitro or in vivo tests that can measure bactericidal activity post-BCG vaccination are surprisingly nonexistent, and no immune correlate of protection and no reliable test of a response to vaccination have been identified. The tuberculin skin test, historically used as an indicator of infection and response to BCG vaccination, correlates poorly with BCG vaccination and does not reliably predict immunity (Fine et al. 1986, 1994). The FDA has approved two assays that measure IFN-γ release from *M. tuberculosis*-specific T cell: the QuantiFERON®-TB Gold In-Tube assay (GFT-G-IT; Cellestis Ltd, Australia) and the T-SPOT.TB® assay (Oxford Immunotec Ltd, UK). These assays show some improvement in detecting active tuberculosis compared to tuberculin tests, but cannot reliably rule out active tuberculosis or reliably distinguish between active or latent infection (Diel et al. 2011; Sester et al. 2011). Unlike the tuberculin skin test, assays that measure IFN-γ appear to be little influenced by prior BCG vaccination (Leyten et al. 2007).

Perforin and granulysin are examples of molecules derived from $CD4^+$ and $CD8^+$ T cell that display bactericidal activity against intracellular pathogens (Canaday et al. 2001; Ernst et al. 2000; Woodworth et al. 2008). Upregulation of granulysin and perforin expression on $CD4^+$ memory T cells by infection or BCG vaccination suggests a potential role as biomarkers of infection or response to vaccination, but cannot be hold as correlates of protection (Capinos Scherer et al. 2009; Mueller et al. 2011).

5.2.5 Is There a Future for BCG?

Currently, BCG remains the only vaccine available for the prevention of tuberculosis. The global effectiveness of its use is impossible to calculate. BCG does not prevent the development of latent infection or reactivation and is therefore unlikely to have halted global transmission of *M. tuberculosis* in any tangible way. However, the effectiveness of BCG in preventing childhood deaths from severe, rapidly progressive disease has been repeatedly demonstrated and justifies its continued use. The challenge remains to develop a vaccine that can induce sterilizing immunity in the human host, which has thus far not been feasible in animal challenge models.

The interests and undertakings in the development of new and more effective vaccines against tuberculosis are intense. Recently, MVA-Ag85A failed for efficacy, and the future of any of the current experimental vaccines is unsure. However, even the availability of new vaccines is unlikely to spell the demise of BCG, and its use in neonates may continue alongside new vaccines in prime-boost scenarios.

5.2.6 Nonspecific Beneficial Effects of BCG

There are indications that BCG has nonspecific beneficial effects on reducing overall childhood mortality in low-resource settings with high childhood mortality rates (Aaby and Benn 2012; Roth et al. 2005, 2006). This effect is thought to be related to the maturational effect of BCG on the neonatal innate immune system. An immunological explanation has recently been offered by Netea and colleagues (Kleinnijenhuis et al. 2012): in a series of human, animal, and in vitro studies, BCG induced long-lasting epigenetic changes that led to increased proinflammatory immune responses against challenges with unrelated antigens. Indeed, in mice BCG led to a 70 % reduction in mortality from an unrelated infection (*Candida albicans* infection) when challenged 2 weeks later. Two trials of early BCG vaccination are planned in Denmark and Australia to study potential nonspecific beneficial effects on morbidity in young children in high-income settings.

BCG is also licensed and used (intravesically) as a therapy for, and prophylaxis against, recurrent tumors in patients with carcinoma in situ (CIS) of the urinary bladder, and to prevent recurrence of Stage TaT1 papillary tumors of the bladder at high risk of recurrence (product is sold under the trade name TICE® BCG: the TICE strain was developed at the University of Illinois from a strain originated at the Pasteur Institute). Although the precise mechanism of action is unknown, it further stipulates BCG's immune stimulating effects.

5.2.7 Vaccines in Development

5.2.7.1 Live-Attenuated Vaccines

There are currently two approaches taken in the development of a live-attenuated tuberculosis vaccine. One approach is to develop an improved BCG strain using genetic modifications, aiming to increase protection by enhancing certain aspects of the immune response, thus providing a more predictable and durable response. The second approach has been to develop a live-attenuated *M. tuberculosis* strain, thereby overcoming potential epitopic differences between the BCG strain and *M. tuberculosis.* Live-attenuated vaccines may have proved to be particularly useful in vaccination of symptomatic HIV-positive children who are at risk of tuberculosis but in whom administration of BCG is contraindicated due to the significant risk of disseminated BCG disease (World Health Organization 2004, 2010). Live-attenuated vaccines in development are summarized in Table 5.1.

A recombinant BCG30 strain that overexpressed the 85B antigen improved survival in guinea pigs and was immunogenic in a Phase I study in humans (Hoft et al. 2008), but is currently on hold.

VPM1002 is a recombinant BCG Prague strain that expresses listeriolysin and which carries a urease deletion mutation to facilitate listeriolysin activity (Grode et al. 2005). This candidate enhances $CD8^+$ T-cell activity and is currently being

Table 5.1 Live-attenuated vaccine candidates under development for prevention of tuberculosis

Candidate	Manufacturer	Strain	Characteristic	Development phase
Recombinant BCG strains for primary vaccination				
VPM1002	Vakzine Projekt Management GmbH	Recombinant BCG Prague	Enhanced listeriolysin activity	Phase II
rBCG30	Aeras	Recombinant BCG Tice	Overexpression of *M. tuberculosis* 85b antigen	Reached Phase I, currently on hold
AERAS-422	Aeras	Recombinant BCG Danish	Expresses perfringolysin; overexpression *M. tuberculosis* 85a, 85b, and Rv3407 antigens	Phase I: halted due to safety concerns
Attenuated M. tuberculosis strains for primary vaccination				
MTBVAC01	Biofabri	Genetically attenuated S02	Inactivated phoP gene	Preclinical
mc(2)6030	–	Genetically attenuated	RD1 and panCD gene deletions	Phase I planned
mc(2)6020	–	Genetically attenuated	lysA and panCD gene deletions	Phase I planned
mc(2)6435	–	Genetically attenuated	Simian immunodeficiency virus Gag expression; attenuated panCD and leuCD	Preclinical
Whole-cell vaccines for therapeutic or prophylactic use				
M. vaccae	–	Inactivated		Phase III complete
M. smegmatis	–	Recombinant attenuated		Not known

investigated in a Phase II study in infants in South Africa (www.clinicaltrials.gov NCT 01479972).

AERAS-422 is a recombinant BCG Danish strain that expresses perfringolysin and overexpresses three protective *M. tuberculosis* antigens. AERAS-422 was shown to be more immunogenic in mice than the parent BCG strain (Sun et al. 2009). A Phase I study (www.clinicaltrials.gov NCT01340820) in humans was recently halted due to side effects (Kupferschmidt 2011) but no additional details are available at the time of writing.

Several groups have attempted to develop live genetically attenuated strains of *M. tuberculosis* of which three are most advanced. MTBVAC01 is an attenuated MT103 strain with an inactivated phoP gene, that is required for intracellular growth of *M. tuberculosis* (Perez et al. 2001). MTBVAC01 is attenuated in preclinical testing and reduced bacterial counts in lung and spleen after intravenous

challenge in animal models (Cardona et al. 2009; Martin et al. 2006; Verreck et al. 2009). Unlike the parent strain, MTBVAC01 does not induce host cell apoptosis (Aporta et al. 2012). Phase I studies were expected to start in 2012 (http://www.newtbdrugs.org/blog/announcement-promising-tb-vaccine-candidate-progresses-to-phase-i-clinical-trials/).

Two mutant strains of *M. tuberculosis* (mc22020 and mc22030) each contain double gene deletions that restrict their growth: mc22030 (DeltaRD1 DeltapanCD) improved survival against tuberculosis in mice (Sambandamurthy et al. 2006, 2005), and both mc22020 (DeltalysA DeltapanCD) and mc22030 administered to macaques showed intermediate levels of protection against respiratory challenge with tuberculosis that was less than that afforded by BCG (Larsen et al. 2009).

5.2.8 Whole-Cell Inactivated Vaccines

Infection with *M. tuberculosis* itself does not convey immunity: that is, both reactivation and reinfection can occur after initial exposure. Whole-cell preparations of *M. tuberculosis* or other *Mycobacterium* are being investigated primarily as therapeutic vaccines for use as an adjunct to chemotherapy.

An inactivated whole-cell vaccine of *Mycobacterium vaccae* has shown mixed results as a therapeutic vaccine for active tuberculosis to date; with evidence of enhanced clearance of bacilli from sputum and improvement in radiological lesions. Evidence of an immunological benefit over chemotherapy was inconclusive (Yang et al. 2011). A Phase III study conducted in Tanzania that evaluated a five-dose prophylactic series of inactivated whole-cell *M. vaccae* administered to BCG-primed HIV-positive adults showed 48 % protection against the development of disseminated tuberculosis (not statistically significant) and 39 % against "definite" tuberculosis (von Reyn et al. 2010).

A live genetically attenuated strain of *Mycobacterium smegmatis* has also been investigated, inducing strong bactericidal immune responses, that were dependent on $CD4^+$ memory T cells, against *M. tuberculosis* in mice (Sweeney et al. 2011).

5.2.9 Conclusions

M. tuberculosis has caused human disease for thousands of years and remains a major public health issue. Despite the efforts of a century of investigation, no broadly effective prophylactic vaccine currently exists. BCG has been used globally for almost 100 years and has had important impacts on childhood morbidity and mortality from severe, rapidly progressive tuberculosis. However, BCG has had little, if any, impact on global disease control. Nevertheless, BCG has an established safety record and its use is likely to continue in the long term either as a standalone vaccine or as a component of prime-boost strategies using new vaccines.

5.3 Pertussis Vaccines

5.3.1 Pertussis: Overview of Bacteriology and Pathology

Bordetella pertussis is a fastidious, aerobic gram-negative bacillus that was first identified in culture in 1906 by Bordet and Gengou. The bacterium causes respiratory infections ("whooping cough") in humans that are characterized by prolonged cough ("the hundred days cough"). In young children, disease manifests as a severe paroxysmal cough with an inspiratory whoop. *B. pertussis* is highly transmissible and acquired directly via the respiratory secretions of close contacts. The disease begins with mild upper respiratory tract symptoms that last 1–2 weeks before onset of the paroxysmal stage, which is characterized by intermittent severe spasmodic coughing episodes accompanied by vomiting or cyanosis, lasting 4 to 6 weeks. The cough gradually resolves during the convalescent phase, which may continue for many months. Complications, including secondary pneumonia, encephalopathy, or neurologic damage arising from hypoxia, are more likely to occur in infants than in older individuals and may be fatal (Cortese et al. 2008; Crowcroft et al. 2003; Stojanov et al. 2000). In older age-groups, who often have past exposure to *B. pertussis* or have been vaccinated, the disease is generally more mild, paroxysms infrequent and the inspiratory whoop absent, leading to delayed or missed diagnoses. Complications in adults include pneumonia, rib fracture, and incontinence. The morbidity and health economic costs associated with pertussis disease in all age-groups are sizeable (De Serres et al. 2000; Lee et al. 2004; Pichichero and Treanor 1997).

Pertussis vaccines have been available since early in the twentieth century and have been highly effective in reducing childhood morbidity and mortality due to pertussis, as illustrated by longitudinal data from the UK (Fig. 5.1) and Sweden (Fig. 5.2). Due to efforts increasing pertussis vaccination coverage worldwide (estimated at 85 % in 2011 (World Health Organization 2012b)), deaths due to whooping cough more than halved between 1990 and 2010, from an estimated 166,500 cases in 1990 to 81,400 cases in 2010 (age-standardized death rate declined from 2.3/100,000 to 1.1/100,000) (Lozano et al. 2012). Pertussis vaccines fall into two broad categories: whole-cell vaccines consisting of suspensions of killed *B. pertussis* (Pw), and acellular vaccines (Pa) containing between one and five purified *B. pertussis* components that induce an immune response and are believed to contribute to pathogenesis. Acellular pertussis vaccines were first introduced in Japan in 1981 and then by most high-income countries in the 1990s because of their improved reactogenicity profile compared with whole-cell vaccines (Zhang et al. 2011).

B. pertussis is only found in humans and no animal reservoir is known to exist. Pertussis is therefore a potentially eradicable disease. However, features of the available prophylactic vaccines and of the disease itself mean that eradication has not yet been possible. In vaccinated populations, *B. pertussis* infections may go unrecognized because of the absence of classical symptoms such as the inspiratory

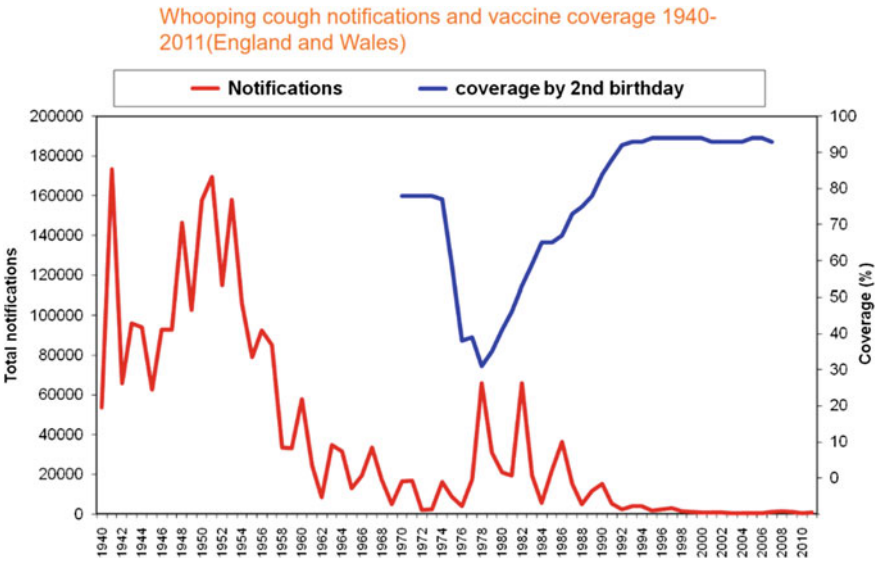


Fig. 5.1 Whooping cough notifications and vaccine coverage 1940–2011 (England and Wales). Reproduced from http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1317136329151. Data from the UK Health Protection Agency

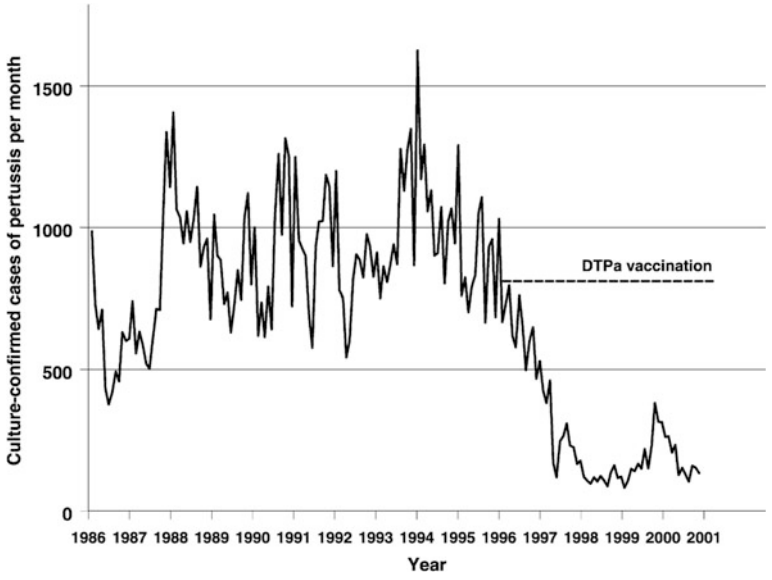


Fig. 5.2 Culture-confirmed and PCR-confirmed pertussis cases: Sweden 1986–2000. Reproduced with permission from Elsevier (Olin et al. 2003)

"whoop"; laboratory diagnosis may be challenging with cultures usually turning out negative after the cough has been present for several weeks; and protection and immunity after infection or vaccination are not lifelong, with natural infections inducing the longest lasting protection, followed by whole-cell vaccines and the shortest lived protection by acellular pertussis vaccines (Hallander et al. 2011; Sheridan et al. 2012). Together these features mean that today, pertussis transmission and circulation continues unabated in all age-groups, including populations with high vaccine coverage. More positively, a greater awareness among clinicians of atypical pertussis presentations, improved diagnostic techniques including polymerase chain reaction (PCR), improved booster vaccination policies that now target older age-groups, parents, and pregnant women, in addition to infants and children, and ongoing vaccine development activities, mean that improved pertussis control may be feasible.

5.3.2 Licensed Vaccines

5.3.2.1 Whole-Cell InactivatedVaccines

Production and testing of killed whole-cell pertussis vaccines began in the early 1920s, soon after the bacterium was first isolated and identified as the causative agent of whooping cough by Bordet and Gengou in 1906 (Pittman 1991). In the 1940s, pertussis vaccines were combined with diphtheria and tetanus toxoids (DTPw) for use in infant immunization programs in an ever growing number of countries (Burgess and Forrest 1996; Centers for Disease and Prevention 2002; Therre and Baron 2000). Due to extensive efforts and investment by the World Health Organization and the Global Alliance for Vaccines and Immunization (GAVI), infant coverage of three doses of pertussis vaccine (whole-cell or acellular pertussis, combined with diphtheria and tetanus) in 2011 was estimated at 85 % (World Health Organization 2012b). Most of the world's unvaccinated children reside in Southeast Asia, Africa, and Oceania.

Over the decades of whole-cell pertussis vaccine use there have been several periods during which vaccination in some countries has ceased or decreased to very low levels. In the 1970s, the safety of whole-cell pertussis vaccines was widely questioned and public acceptance of vaccination fell. In Japan, infant vaccination was suspended in 1975 for 6 years after publicity linking pertussis vaccination with infant deaths. Over this period pertussis incidence increased from 0.2 to 11.3 cases per 100,000 individuals (Sato and Sato 1999a). In Japan, these events initiated intense research and development of the first acellular vaccines, which were introduced in 1981 (Sato and Sato 1999b). In the UK, public rejection of vaccination led to a precipitous drop in infant vaccination coverage from 75 % in 1974 to 30 % in 1978 (Pollard 1980). This was followed by the reappearance of major pertussis outbreaks in the UK: between 1977 and 1979 there were almost 100,000 reported pertussis cases, with the highest notification rates in regions with the lowest

vaccination coverage notification (Pollard 1980) (Fig. 5.1). Confidence in pertussis vaccination took over a decade to be restored, but coverage in the UK gradually increased to a new high by the 1990s, with associated decreases in pertussis notifications (Fig. 5.1). In Sweden, where the whole-cell pertussis vaccine in use was withdrawn in 1979 and an acellular pertussis vaccine was not introduced until 1996, regular pertussis epidemics occurred during the intervening period (Fig. 5.2), with the incidence of pertussis decreasing from 89–150 per 100,000 to 17–26 per 100,000 after reintroduction of infant (acellular pertussis) vaccination (Olin et al. 2003). These events in Japan, the UK, and Sweden serve to highlight the benefits of continued pertussis vaccination in preventing acute pertussis disease in vulnerable populations, and in dampening epidemic activity. The initial concern that whole-cell pertussis vaccination might be linked to encephalopathy and Sudden Infant Death Syndrome was eventually proven to be highly unlikely and not demonstrable (Fleming et al. 2001; Gale et al. 1994; Griffin et al. 1990; Heininger 2010; Hoffman et al. 1987; Miller et al. 1993, 1981; Moore et al. 2004; Walker et al. 1988).

Despite the introduction of acellular pertussis vaccines in countries with established economies, today whole-cell pertussis vaccines are still used to immunize and protect the vast majority of the world's birth cohorts against whooping cough, most of whom reside in the developing world (Fig. 5.3).

Tests of potency. Whole-cell pertussis vaccines are suspensions of *B. pertussis* grown in liquid or solid media and inactivated by several methods including formalin, other chemical means, or high temperature. Manufacturing procedures have changed very little since early in the twentieth century when the first vaccines were developed.

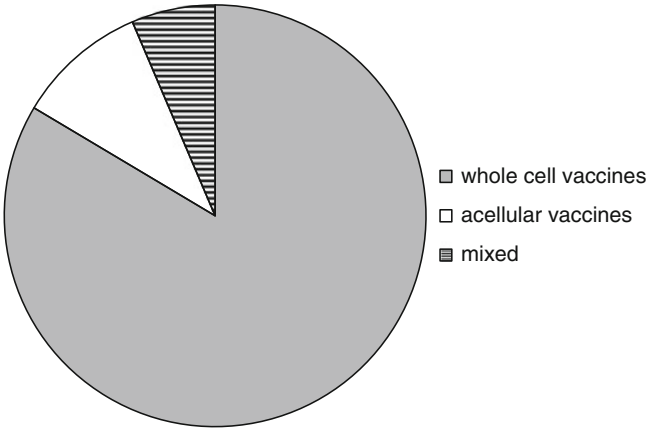


Fig. 5.3 Global pertussis vaccine use. 83.3 % of the world's annual birth cohort is born in nations offering whole cell pertussis vaccines (*grey*) in their pediatric immunization programs; 10 % is offered acellular pertussis vaccine (*white*); and 6.4 % a combination of whole cell and acellular vaccines (*grey/white*). *Source* Data on immunization schedules derived from annual WHO/UNICEF Joint Reporting Form (update of 15 December 2010 (www.who.int/entity/immunization_monitoring/data/schedule_data.xls). Birth cohort by country estimated from 2008 country-specific population and birth rates from www.NationMaster.com

There are pronounced differences in vaccine composition between manufacturers. This fact, as well as the varying levels of impurities such as lipopolysaccharide (LPS) in individual products, affects reactogenicity and efficacy (Cherry 1996; Galazka 1993). Estimates of clinical efficacy as well as immune responses to specific antigens have proven to vary considerably between different whole-cell vaccines (Edwards et al. 1991; Fine and Clarkson 1987; Steinhoff et al. 1995).

The manufacturing of whole-cell pertussis vaccines is complicated because of the risk that the expression of virulence/protective components may be downregulated in vitro, as a result of shifting to the avirulent phase. Manufacturers who are tempted to focus on high biomass levels during fermentation processes may risk generating *B. pertussis* cultures that express low levels of protective components. This risk is not mitigated by way of a reliable potency assay, since the only required potency assay (the Kendrick mouse intracerebral challenge assay) does not prevent the release of poorly efficacious whole-cell pertussis vaccines. The technically demanding Kendrick test was shown to correlate with clinical efficacy in studies conducted in the UK, and was adopted by the World Health Organization for the standardisation of potency in 1964, distinguishing between potent and subpotent batches (Pittman 1991). However, this test has since shown inconsistent correlation with immunogenicity and efficacy in humans. This was evidenced during the 1990s' National Institute of Health-sponsored efficacy trials when Connaught's whole-cell pertussis vaccines (that passed the Kendrick potency assay) demonstrated unexpectedly low efficacy (Greco et al. 1996; Gustafsson et al. 1996). A comparable situation occurred in the Netherlands, where a drop in potency (suspected retrospectively) of a nationally manufactured whole-cell vaccine remained unnoticed during the manufacturing and release process and was only detected in the field when pertussis cases started to rise alarmingly in the country (de Melker et al. 1997; Health Council of the Netherlands 2004). Poor effectiveness of a whole-cell vaccine were also documented in Canada and Sweden, where pertussis incidence rates decreased once a more effective acellular vaccine was introduced (Skowronski et al. 2002; Olin et al. 2003). The most recent evaluations of the efficacy of some whole-cell vaccines occurred in the 1990s in the context of efficacy trials for the acellular pertussis vaccines, showing that in the short-term the better whole-cell pertussis vaccines performed as well, or even better, than acellular pertussis vaccines, but some seriously underperformed [reviewed in Storsaeter et al. (2007), Zhang et al. (2012)].

The examples above are evidence of lack of control of vaccine quality and question the adequacy of the mouse intracerebral challenge test as an indication of vaccine potency. Few currently available whole-cell pertussis vaccines have been formally evaluated for efficacy in clinical trials, and given the lack of an immunological correlate of protection and that many countries using whole-cell vaccines lack comprehensive surveillance systems, the risks of failing to identify low efficacy of the whole-cell vaccine are substantial. This includes the risk that the currently used Kendrick test may fail to identify loss of potency, leaving manufacturers of initially effective whole-cell vaccines at risk of losing potency and of maintaining poor *B. pertussis* working seeds.

A candidate alternative model to the Kendrick intracerebral challenge model is the mouse intranasal challenge model (Corbel et al. 2004; Corbel and Xing 2004). This model reproduces clinical efficacy estimates of licensed whole-cell and acellular pertussis vaccines observed in clinical trials and could replace or complement the intracerebral challenge test (Denoel et al. 2005; Guiso et al. 1999). In addition, considering that for whole-cell vaccines IgG antibodies against fimbriae (FIM) are associated with agglutination and hence are considered to be the most important mechanism of action of these vaccines, the use of the (FIM) agglutination test as an additional in vitro tool to test release batches could also be considered. Discussion and decision making are needed on the implementation of new whole-cell pertussis vaccine potency tests that are better in discriminating between vaccines of different efficacies than the currently used intracerebral mouse test. There is a particular need for a more discriminatory test for low-income countries that do not yet have comprehensive surveillance systems in place, in order to facilitate the purchase and use of vaccines with acceptable levels of efficacy.

Manufacturers and vaccines. The development of a wide range of combination vaccines using acellular pertussis components has facilitated vaccine acceptance and uptake in high-income countries. By contrast, development of broad combination vaccines that include a whole-cell pertussis component lagged until recently. At the time of writing there are four manufacturers providing fully liquid whole-cell pentavalent vaccine combinations (diphtheria, tetanus, whole-cell pertussis, hepatitis B, and *Haemophilus influenzae* type b: DTPw-HB-Hib) with World Health Organization prequalification status (a prerequisite for use in many developing countries lacking well resourced regulatory agencies) (Table 5.2).

Limitations of pertussis vaccines. Since the 1940s, pertussis vaccines have been instrumental in preventing morbidity and mortality due to pertussis in children. Suboptimal potency and variable immunogenicity and efficacy notwithstanding, there are other reasons why currently available pertussis vaccines, whole-cell or acellular, have been unable to halt pertussis transmission or prevent ongoing infection. The most important of these are the low effectiveness of current vaccines in preventing subclinical or mild disease (Zhang et al. 2012), and the short-lived nature of immunity following natural infection and vaccination, estimated to be between 4 and 12 years after vaccination (Grimprel et al. 1996; Salmaso et al. 2001; von Konig et al. 2002), implying that no or low impact on *B. pertussis* transmission can be expected.

Some studies suggest that the duration of protection may be longer after whole-cell than after acellular pertussis vaccination (Lacombe et al. 2004; Lugauer et al. 2002; Sheridan et al. 2012; Wendelboe et al. 2005; Zhang et al. 2012). However, the duration of immunity observed are influenced by the number of doses administered, the vaccination schedule, the nature of vaccine used, herd immune effects, as well as the level of circulating *B. pertussis* in the community under study. Although no correlate of protection has yet been established, whole cell and acellular pertussis vaccines are believed to work and activate the host's immune system differently. Whole-cell pertussis vaccines induce a Th1-directed immune response with activation of complement and production of opsonising antibodies

Table 5.2 Pentavalent whole cell vaccines (diphtheria, tetanus, whole cell pertussis, hepatitis B, and *Haemophilus influenzae* type b conjugate vaccine, with World Health Organization prequalification status (World Health Organization 2012c)

Vaccine	Manufacturer (country)	Presentation
Quinvaxem™	Berna Biotech Korea Corporation (a Crucell Company) (Republic of Korea)	Single-dose vial
		Liquid
Diphtheria–tetanus–pertussis (whole cell)–hepatitis B–*Haemophilus influenzae* type b	Biological E Limited (India)	Single-dose vial or 10-dose vial
		Liquid mixed with lyophilised Hib component
Diphtheria–tetanus–pertussis (whole cell)–hepatitis B–*Haemophilus influenzae /Emphasis> type b*	Biological E Limited (India)	Single-dose vial or 10-dose vial
		Liquid
Tritanrix™ Hib	GlaxoSmithKline Vaccines (Belgium)	Two-dose vial
		Liquid mixed with lyophilised Hib component
Tritanrix HB+Hib	GlaxoSmithKline Vaccines (Belgium)	Single-dose vial
		Liquid mixed with lyophilised Hib component
Zilbrix™-Hib	GlaxoSmithKline Vaccines (Hungary)	Single-dose vial
		Liquid
Diphtheria, tetanus, pertussis, hepatitis B, and *Haemophilus influenzae* type b conjugate vaccine adsorbed	Serum Institute of India Ltd (India)	Single-dose vial, two-dose, and 10-dose vial
		Liquid mixed with lyophilised Hib component
Diphtheria, tetanus, pertussis, hepatitis B, and *Haemophilus influenzae* type b conjugate vaccine adsorbed	Serum Institute of India Ltd (India)	Single-dose vial, two-dose, and 10-dose vial
		Liquid

Hib Haemophilus influenzae type b

that contribute to bacterial clearance, with responses to fimbriae playing a key role (Ausiello et al. 1997; Ryan et al. 1998). Acellular vaccines induce a mixed Th1/Th2 response and it is thought that their mode of action relies more on preventing adhesion of bacteria to epithelial surfaces and inducing neutralization of pertussis toxin (Weingart et al. 2000). Pertussis toxoid is typically found in low levels in whole-cell vaccines, but comprises the principal component in acellular pertussis vaccines. By contrast, most acellular pertussis vaccines do not include fimbriae that are responsible for the whole-cell associated agglutination responses. The long-term implications of these differences are not understood. One study in mice given whole-cell or acellular pertussis vaccines showed prolonged efficacy against respiratory challenge with *B. pertussis*, even when antibodies had decreased to undetectable levels, suggesting induction of memory cells by both vaccines (Mahon

et al. 2000). However, long-term bacterial clearance was significantly higher in whole-cell vaccinated mice than in mice who received acellular vaccine (Mahon et al. 2000), as also demonstrated more recently in a retrospective human cohort study, where just one priming dose of killed whole-cell pertussis vaccine was linked to longer durability of protection (Sheridan et al. 2012; Liko et al. 2013).

As immunity wanes into adolescence and adulthood, individuals become susceptible to subclinical or clinical infections that are then transmitted to family members, with high risk of adverse outcomes for very young, unvaccinated, or partially vaccinated infants (Crowcroft et al. 2003; de Greeff et al. 2012; Edwards 2005). The failure of both whole-cell and acellular pertussis vaccines to arrest transmission is evident in the evolving epidemiology of the disease, where disease incidence peaks are observed in adolescents and adults, in addition to their young unvaccinated infant contacts (Campbell et al. 2012; Winter et al. 2012; Zepp et al. 2011).

Is there a future for whole-cell pertussis vaccines?Whole-cell pertussis vaccines have the advantage of low production costs, higher immunogenicity and adjuvant effect (Vogel et al. 1987), and longer duration of protection compared to acellular vaccines (Sheridan et al. 2012; Witt et al. 2012). Furthermore, high efficacy whole-cell vaccines may be more effective than the highest efficacy acellular vaccines (Sheridan et al. 2012; Zhang et al. 2012). The major disadvantages of whole-cell pertussis vaccines relate firstly to their greater local and systemic reactogenicity compared to acellular vaccines (Jefferson et al. 2003; Zhang et al. 2011), which seems to increase with the more vaccine doses received, making them unsuitable for use in adolescents and adults. However, decades of use and intensive epidemiological investigations have proven the safety of whole-cell pertussis vaccines (Howson and Fineberg 1992). The second disadvantage is the current limitation in potency testing methods, meaning that there is an ongoing risk that suboptimal whole-cell pertussis formulations are being administered to infants worldwide. An alternative standardized measure of potency, like the mouse intranasel challange model, is needed.

Pentavalent combination vaccines containing whole-cell pertussis are becoming the current standard of care in developing countries. As global poliomyelitis eradication becomes a reality, immunization with low-cost inactivated poliovirus (IPV) vaccine incorporated into pediatric combination vaccines (preferably hexavalent; protecting against diphtheria, tetanus, pertussis, hepatitis B, *H. influenzae* type B, and IPV) using whole-cell pertussis vaccines may be the preferred way to ensure high coverage of all antigens. However, development of whole-cell pertussis and IPV vaccine combinations is hindered by an anticipated interaction between IPV and thiomersal. In the 1950s, thiomersal used as preservative in a combined DTPw and IPV vaccine was described as "detrimental to the poliomyelitis virus" (Davisson et al. 1956). More recently, the effect of thiomersal on IPV potency under conditions of long-term storage at +4 °C was reinvestigated. Loss of potency of type 1 polio virus antigen to undetectable levels after 4–6 months was reported, whereas for types 2 and 3 the potency loss was within the expected range (not more than 10 % loss per year of storage) (Sawyer et al. 1994). Thiomersal is used during the production of whole-cell pertussis vaccines to inactivate the bacterium and toxins and to maintain a sterile production line.

Although thiomersal is largely removed following the purification procedure, traces of thiomersal remain present. In addition, thiomersal is added back as a preservative in multidose vials. The National Institute of Public Health and Environmental Protection (RIVM) in the Netherlands developed a combined DTPw and IPV vaccine in 1962 that used 2-phenoxyethanol rather than thiomersal as preservative. This vaccine was tested in clinical trials in Africa (Rumke et al. 1993). A combined DTPw, IPV, and *H. influenzae* type b vaccine that also used 2-phenoxyethanol as preservative was produced by Sanofi Pasteur (Pentacoq) (iDrugInfo.com 2000–2012). A hexavalent whole-cell pertussis vaccine combination containing IPV was recently shown to be immunogenic in infants (Quiambao et al. 2012). For currently licensed vaccines, substituting thiomersal with an alternative preservative has the potential to modify the stability, safety, and effectiveness of these vaccines. The replacement of thiomersal as a vaccine preservative would therefore require manufacturers to establish through nonclinical and clinical trials that the vaccine when formulated with the substitutive preservative is noninferior in its safety and effectiveness.

Although a first priming with killed whole cell pertussis vaccines appears beneficial (Sheridan et al. 2012; Liko et al. 2013), countries currently using acellular pertussis vaccines in infants are unlikely to return to using whole-cell pertussis vaccines since this would require public acceptance of higher levels of reactogenicity in infants. However, whole-cell pertussis vaccines combined with other vaccine antigens of other pathogens will likely remain the vaccine of choice for infants in low-income countries because of the lower cost, and superior duration of protection as compared to acellular pertussis vaccines.

5.3.3 Vaccines in Development

5.3.3.1 Live-AttenuatedPertussis Vaccines

Despite the enormous impacts that pertussis vaccines have had in preventing morbidity and mortality in children due to pertussis, better vaccines are needed (Poolman et al. 2011; Storsaeter and Wolter 2006). Development of live-attenuated pertussis vaccines administered via the intranasal route has been ongoing since the 1990s. Live-attenuated vaccines aim to replicate natural infection, potentially inducing broad mucosal and systemic immunity against multiple virulence factors, and potentially resulting in improved bacterial clearance and longer lasting immunity. Two live-attenuated candidates have been evaluated in animal models. The most advanced live-attenuated vaccine candidate is BPZE1, which has been genetically detoxified by altering or replacing genes for pertussis toxin, tracheal cytotoxin, and dermonecrotic toxin (Locht et al. 2004; Mielcarek et al. 2006b). BPZE1 showed high efficacy in protecting infant and adult mice against respiratory challenge by *B. pertussis* and related species, and appeared to induce durable immunity (Feunou et al. 2010b; Kammoun et al. 2012; Skerry et al. 2009). Passive transfer of antibodies

to unvaccinated mice resulted in bacterial clearance (Skerry et al. 2009). Immunity appeared to be dose-dependent and was thought to be due to the generation of humoral responses and induction of $CD4^+$ T cells (Feunou et al. 2010a; Mielcarek et al. 2010). BPZE1 is proposed for use as a single intranasal dose for infants as young as at the day of birth (Mielcarek et al. 2006a). A Phase I clinical study in adults with BPZE1 has recently been completed and demonstrated that BPZE1 was safe and induced pertussis-specific IgG responses in those participants in whom intranasal immunization resulted in nasopharyngeal colonization (approximately 40 %) (www.clinialtrials.gov NCT01188512) (Locht and Mielcarek 2012). BPZE1 is also being investigated for utility as a carrier for other antigens (Ho et al. 2008).

Another live-attenuated pertussis vaccine candidate in early development is the *aroQ* mutant strain. Deletion of the *aroQ* gene results in reduced propagation capacity of the bacterium. A single intranasal dose induced humoral and cell-mediated responses in mice, and protection against respiratory challenge (Cornford-Nairns et al. 2012).

Licensure of a live-attenuated vaccine would represent the first major development in pertussis vaccination since the availability of acellular pertussis vaccines around 30 years ago. Live-attenuated vaccines could potentially be given very early in life, giving protection to an age group at most risk of severe disease and death due to pertussis. However, the potential for reversion and safety aspects associated with live-attenuated vaccines, in particular when administered intranasally, will need to be carefully monitored. Furthermore, very little is known about the principle of mucosal colonization of the upper respiratory tract by commensals shortly after birth and the activation of early immune maturation and how this may be disrupted or enhanced by intranasal *B. pertussis* immunization. Furthermore, the extent and durability of immune response in humans remains unknown. Indeed, since immunity is not lifelong after natural infection, lifelong immunity after vaccination may not be possible to be achieved.

5.3.4 Conclusion

Whole-cell pertussis vaccines were some of the first vaccines to be widely used in children, and are one of the oldest vaccines still in use. Extensive experience over many decades confirms the safety and effectiveness of whole-cell pertussis vaccines in preventing pertussis disease and deaths in children. Limitations of vaccination including incomplete and short duration of protection, and the lack of any available vaccine for use in neonates in whom the disease is most severe, remain to be overcome. Recently, several high-income countries (including but not limited to the UK, the US, and France) have started to recommend and practise immunization of pregnant women with acellular pertussis as a mean to protect newborns against pertussis infections in the first 3 crucial months in life. The efficacy of maternal immunization and the risk for potential interference with subsequent infant acellular pertussis vaccine priming is at this moment in time still unknown.

In view of the shorter duration of protection induced by acellular than whole-cell pertussis vaccines, as well as the lower cost of whole-cells vaccines over acellular vaccines, it is anticipated that the WHO Strategic Advisory Group of Experts (SAGE) for low-income countries will continue to recommend the use of whole-cell vaccines (World Health Organization 2005). Thus, whole-cell pertussis vaccines now incorporated into pentavalent and soon to be hexavalent vaccines are likely to remain the standard in low-income countries for years to come.

5.4 Cholera Vaccines

5.4.1 Cholera: Overview of Bacteriology and Pathology

Cholera is a severe acute diarrheal disease caused by infection of the small intestine with the gram-negative bacterium *Vibrio cholerae.* Humans are the only vertebrate hosts and transmission to humans is by drinking water in which *V. cholerae* either occurs naturally or that has been contaminated by feces of an infected person, or, less frequently, by eating contaminated fish and shellfish. The risk of localized cholera outbreaks is high due to the highly variable incubation period of *V. cholerae* that can be as short as 2 h and as long as 5 days. As a consequence many individuals may become infected before the source of contamination is identified. It is estimated that there are 3–5 million cholera cases and 100,000–120,000 deaths due to cholera each year.

V. cholerae is one of the most common organisms in surface waters of both marine and freshwater habitats. Between epidemics, viable *Vibrios* are present in water surfaces predominantly in a dormant, nonculturable state, and are able to form biofilms that provide a microenvironment facilitating environmental persistence (Alam et al. 2007). *Vibrios* "re-emerge" after years of seeming absence when a combination of favorable environmental conditions appears right. Attached to plankton that act as hosts for the dormant cholera organism, *Vibrios* can travel long distances either by hitchhiking with plankton on ocean currents across thousands of kilometers and over periods of months and years, or by means of ballast water of cargo ships crossing the world's oceans (Rivera et al. 2012). Alternatively, it has been suggested that *Vibrios* can also travel through air as hitchhikers attached to the surface of adult nonbiting midges (observed mainly with *Chironomus* sp.) that are carried by the wind and that transmit the bacteria from one body of water to another over large areas (Paz and Broza 2007).

V. cholerae is a noninvasive human pathogen that colonizes the small intestine and produces an enterotoxin, cholera toxin (CT) that activates adenylate cyclase in intestinal epithelium cells, converting these cells into pumps that extract water and electrolytes from blood and tissues, pumping it into the lumen of the intestine. The clinical result is profuse, painless, watery diarrhea that can quickly lead to severe dehydration and death if treatment is not given promptly. Mortality rates for untreated cholera bare as high as 50–60 % (Harris et al. 2012). Oral Rehydration

Therapy, which involves the drinking of a solution of salts and sugars (30 ml sugar: 2.5 ml salt: 1 l of water) is a simple treatment to avert cholera deaths, provided that noncontaminated drinking water is available. It is well established that antibodies to *V. cholerae* LPS confer protection against disease, probably by sterically hindering the bacteria from adhering to the mucosal surface. In addition, antibodies directed against CT can prevent disease. Cholera toxin consists of one toxic A subunit and five identical B subunits. The B subunit (CTB) is the binding portion of the toxin and it is nontoxic in itself. Anti-CT antibodies are almost exclusively directed against the CTB portion and prevent the toxin from binding and thereby limit the symptoms of disease. Antibody responses against CTB and *V. cholerae* LPS are believed to act synergistically rather than additively (Svennerholm and Holmgren 1976).

There are currently 139 known *V. cholerae* serogroups (classified based on LPS-O antigens); however, only two, serogroups O1 and O139 cause outbreaks (World Health Organization 2001). Until 1992, when the O139 Bengal strain emerged in Bangladesh, O1 had been the only serotype known to cause epidemic cholera, and still causes the majority of outbreaks. *V. cholerae* serogroup O1 has two biotypes; classical and El Tor (the latter can be distinguished from classic biotypes by the production of haemolysins). Each biotype has three distinct serotypes; Ogawa, Inaba, and Hikojima (O antigen factors AB, AC, and ABD, respectively). Serogroup O139 is currently confined to the Southeast Asian region and is thought to be a more virulent serogroup variant of the El Tor biotype. O139 and the O1 El Tor strains are closely related in most parts of their genomes but carry different O antigen genes, suggesting the transfer of O139-specific genes from an unknown donor into a recipient El Tor strain (Nair et al. 2002).

Although personal hygiene measures, food safety and sanitation are fundamental to prevent and control cholera, vaccines are also a key preventative, particularly in settings where good hygiene measures are hard to achieve and where cholera epidemics are most likely to occur. Cholera vaccines target three different populations: 1. Travelers into endemic settings (who are mostly immunologically naive); 2. Populations living in areas facing epidemic cholera outbreaks (who are also typically immunologically naive); and 3. Populations living in cholera-endemic areas (who acquire immunity with prolonged duration of exposure, i.e., with increasing age). In practice, immunization against cholera is predominantly used by the first target group: travelers. Nevertheless, following recent cholera outbreaks in Zimbabwe in 2008–2009 and in Haiti in 2010–2011, WHO now recommends immunization with currently available cholera vaccines (in conjunction with the usually recommended control measures) in areas where cholera is endemic or epidemic. At the start of the outbreak in Haiti in October 2010, only one cholera vaccine, *Dukoral*® (SBL Vaccines, Sweden), was licensed and WHO prequalified, with fewer than 300,000 doses available. WHO has since begun stockpiling cholera vaccines in order to be able to immediately respond to an outbreak, initially aiming to stockpile vaccine sufficient to allow vaccination of one million people (World Health Organization 2012d).

5.4.2 Vaccines Licensed and in Use

The cholera vaccines that are currently available are all oral whole-cell killed vaccines. *Dukoral*™ and *Shanchol*™ (Shantha Biotechnics, India) are both liquid, drinkable vaccines, and both are currently WHO prequalified. *OraVacs*™ (Shanghai United Cell Biotechnology Co., Ltd., China) is the only oral inactivated cholera vaccine in capsule form, but is only available to the Chinese market. One live-attenuated oral cholera vaccine has been licensed in the past, CVD 103-HgR: however, production of this vaccine was suspended in 2004.

5.4.3 Whole-Cell Inactivated Vaccines

Since cholera vibrios and the toxin they produce remain localized to the intestinal surface and lumen and exert their action locally on the epithelium, local intestinal immunity is of critical importance for protection. This has favored the concept of oral cholera vaccines as the most efficient way to induce local protective secretory IgA antibody responses (Clemens et al. 2011). Earlier parenteral inactivated whole-cell cholera vaccines had limited efficacy (approximately 50 %) and a low duration of protection (6 months only) on top of a high reactogenicity profile, and are currently no longer used. Even though the human intestinal mucosa generally displays immune tolerance to nonliving foreign material and commensal flora (meaning that exposure does not induce an immune response), oral inactivated cholera vaccines have proven to be very successful in preventing cholera.

Since *V. cholerae* is a strictly human pathogen, there is no valid animal model to predict the protective efficacy of vaccines. Moreover, killed Gram-negative bacteria are not immunogenic when administered by the oral route in animals. Demonstrating the protective effect and immunogenicity of oral whole-cell killed cholera vaccines therefore relies on clinical studies in humans. Although intestinal IgA and IgG responses are probably the best correlate of protection, they are difficult to assess in large clinical studies. Therefore, serum IgG or IgA titres against CTB or cholera LPS, or vibriocidal titres are used as markers of vaccine immunogenicity, and are used for consistency studies and for comparing different preparations or regimens of the same vaccine. Of notice, the rabbit ileal loop technique has been used and discussed as a tool to preclinically evaluate the possible relative protective efficacy of different cholera antigens or vaccines: in this model, the ileal loops of immunized New Zealand rabbits are challenged with graded doses of fully virulent *V. cholerae* bacteria and the dose of bacteria causing 50 % fluid accumulation (ED50) in the loops is determined (Svennerholm 2011).

5.4.4 Vaccines and Manufacturers

Dukoral™. This was originally manufactured by the Swedish National Bacteriology Laboratory (SBL). The vaccine contains a mixture of whole-cell inactivated *V. cholerae* of four different O1 strains, comprising the classical and El Tor biotypes of both the Inaba and Ogawa serotypes, combined with 1 mg recombinant CTB (rCTB) (Table 5.3). The addition of CTB significantly adds to the level of protection induced (Clemens et al. 1990). Whereas the first formulation of the vaccine contained native CTB (purified from *V. cholerae* Inaba 569b), this has been replaced with rCTB in 1992 (a protein of 102 amino acids, produced in *V. cholera* strain 213 of serotype Inaba, biotype Classical, with a delete CT A subunit gene). The rCTB differs from native CTB in that the former contains up to seven extra amino acids at the N-terminal: however, no differences in immunogenicity or safety between rCTB and the previously used native CTB could be detected by direct comparison (Sanchez and Holmgren 1989). Detailed information on CTB can be found in Chap. 6 on Bacterial Subunit Vaccines.

To induce protection, adults require two doses of the vaccine and children 2–6 years of age require three doses, given with an interval of 1–6 weeks. To induce prolonged protection, booster doses should be given to adults every 2 years and every 6 months to children aged between 2 and 6 years of age. This is particularly important in endemic settings. *Dukoral*™ is administered in a bicarbonate buffer to

Table 5.3 Oral inactivated cholera vaccines

	Dukoral™	Shanchol™	Oravacs™
No. *Vibrio*	1×10^{11}	1.5×10^{11}	5×10^{10}
Strains	O1 Inaba Classical, Cairo 48, heat-inactivated (2.5×10^{10})	O1 Inaba Classical, Cairo 48, heat-inactivated (2.5×10^{10})	O1 Inaba Classical
	O1 Inaba El Tor, Phil 6973, formalin-inactivated (2.5×10^{10})	O1 Inaba El Tor, Phil 6973, formalin-inactivated (5×10^{10})	O1 Inaba El Tor
	O1 Ogawa Classical, Cairo 50, heat-inactivated (2.5×10^{10})	O1 Ogawa Classical, Cairo 50, heat-inactivated (2.5×10^{10})	–
	O1 Ogawa Classical, Cairo 50, formalin-inactivated (2.5×10^{10})	O1 Ogawa Classical, Cairo 50, formalin-inactivated (2.5×10^{10})	–
	–	O139, 4260B, formalin-inactivated (5×10^{10})	–
CTB[a]	Recombinant (1 mg)	No	Recombinant (1 mg)
Formulation	Drinkable	Drinkable	Capsule
WHO-PQ	Yes—since 2001	Yes—since September 2009	No

[a] Cholera toxin B subunit

protect the immunogenicity of the acid-labile CTB subunit against stomach acid, and intake of food and drink is recommended to be avoided 1 h before and after vaccination in order to evade increases in gastric acid production.

The shelf life of *Dukoral™* when stored between +2 and +8 °C is 3 years. After dissolving in water and addition of the buffer, the mixture should be drunk within 2 h.

Dukoral™ was the first oral (liquid) inactivated cholera vaccine to be tested in a large field trial in a cholera-endemic country. Of the many clinical trials performed, the efficacy of *Dukoral™* against cholera was established in three pivotal randomized placebo-controlled trials, involving in total 112,639 subjects (30,812 received the assigned number of active *Dukoral™* vaccine doses), and including 13,670 children aged 2–5 years (4,919 received the assigned number of active vaccine doses). Study populations included people from endemic areas (Bangladesh) and epidemic regions (Peru): for the cholera indication no formal efficacy studies have been performed in naive travelers.

Together these clinical studies show that *Dukoral™* affords protective efficacy against cholera in all age-groups in both endemic areas (Bangladesh) and epidemic areas where cholera had not been as prevalent previously (Peru Military). The large clinical trial in Bangladesh (endemic area) clearly established that *Dukoral™* conferred significant protection against cholera (Clemens et al. 1988), demonstrating a short-term protection (6 months) of 85 % in both children and adults, and 60 % protection 2 years postimmunization in adults. In children aged 2–5 years, the duration of protection was much shorter than in the older population, although the initial response to vaccination was similar. For children under 6 years of age, protection in the first 6 months was only 35 % when vaccinated with the whole-cell killed vaccine not containing CTB, but was 100 % for the combined whole-cell and CTB vaccine: thereafter protective efficacy was similar. Overall, vaccine-induced protection against Classical strains was slightly superior, especially during the 2nd year, compared to that against El Tor strains (Clemens et al. 1990).

Due to the similarity between CTB and the heat-labile enterotoxin (LT) of enterotoxigenic *Escherichia coli* (ETEC), *Dukoral™* has the potential to cross-protect against ETEC infections. In clinical studies with the native CTB-containing *Dukoral™*, short-term protection was found in endemic populations in Bangladesh (protection against LT-ETEC diarrhea was 67 %), and in travelers to endemic areas (Finnish travelers to Morocco reported protective efficacy against LT-ETEC diarrhea of 60 % and all traveler's diarrhea of 23 %; the US students in Mexico reported protective efficacy against any ETEC diarrhea of 50 %). Considering that protection is only short-term (3–6 months), the *Dukoral™*-induced protection to ETEC may be considered adequate for travelers who can be revaccinated for each new trip; however, in endemic areas protection is too short-lived to be useful.

Dukoral™ was introduced in Sweden in December 1991, approved in the European Union (EU) through a centralized procedure in April 2004, and is registered worldwide in 37 countries outside the EU. *Dukoral™* is on the WHO list of prequalified vaccines and obtained WHO registration in 2001. *Dukoral™* is indicated for the active immunization against cholera infection of adult and children ≥2 years of age, who will be visiting areas with ongoing or anticipated epidemics,

or who will be staying in areas of risk. In some countries, *Dukoral*™ is also indicated for the active immunization against ETEC of adults and children who will be visiting areas posing a risk of diarrheal disease caused by ETEC.

Shanchol™ (*mROC-Vax*, *Shantha Biotechnics*). This contains *V. cholerae* O1 Inaba El Tor and Classical strains, O1 Ogawa Classical strain, as well as an O139 strain (Table 5.3). The latter makes this vaccine a favorable candidate for immunization in the Southeast Asian region where serogroup O139 is causing cholera outbreaks. However, since *Shanchol*™ does not contain CTB, the vaccine may be less protective compared to *Dukoral*™. Additionally, it contains double the amount of LPS compared to *Dukoral*™. An advantage of *Shanchol*™ over Dukoral is its low cost and hence attractiveness for the endemic market—which was also the main driver for its development.

Shanchol™ was developed from the earlier *ORC-Vax*™: a whole-cell killed oral cholera vaccine that was developed and licensed in Vietnam 1997 for use in its public health program. *ORC-Vax*™ was produced by the Vietnamese government-owned producer VaBiotech as a two-dose course for children ≥1 year old and adults. Between 1998 and 2009 more than 20 million doses were administered to children and adults in high-risk areas of Vietnam. Because the Vietnamese national regulatory authority is not recognized by WHO, the vaccine could not be considered for international use. To serve the wider global community, technology was therefore transferred to Shantah Biotechnics in India, where the vaccine was licensed in 2009 and sold under the trade name *Shanchol*™ as a two-dose vaccine, again for use in individuals ≥1 year of age. In September 2011, *Shanchol*™ received WHO prequalification based on the results of a placebo-controlled clinical trial in India involving 66,900 individuals of ≥1 year of age: the protective efficacy achieved in this trial was 17 % in children <5 years, and 66–81 % in older individuals (Sur et al. 2011). Protective efficacy over a follow-up period of 3 years was 43 % in those <5 years of age, and 61–88 % in older age-groups. Clinical trials for *Shanchol*™ are ongoing and include a Phase IV follow-up study in India to study the immunogenicity and safety of a one-dose versus two-dose booster regimen given 5 years after the initial two doses (NCT01579448); a feasibility and effectiveness study of mass cholera vaccination to reduce *V. cholerae* diarrhea in a high incidence urban area in Bangladesh, in which participants are randomized to receive behavioral intervention in addition to vaccine, vaccine only, or no vaccine nor intervention (NCT01339845); and a Phase II trial evaluating whether in children (1–17 years of age) and adults (≥18 years) a 28-day dosing interval results in improved immune responses to the second dose compared to the 14-day interval (NCT01233362).

Shanchol™ is inexpensive (US$1.25/dose) and does not require a buffer, and is therefore well suited for use in low-income countries. It has a shelf life of 24 months when stored at +2–8 °C.

Oravacs™ (*Shanghai United Cell Biotechnology Co., Ltd.*). Less information is available for *Oravacs*™, which is an oral cholera vaccine consisting of rCTB and inactivated whole-cell *V. cholerae* O1 Classical biotype or El Tor biotype, presented in an enteric-coated capsule. *Oravacs*™ is indicated for protection against cholera and traveler's diarrhea caused by ETEC in children 2 years of age and

older, teenagers, and adults having contact or in risk of contact with the disease. The recommended immunization schedule consists of three doses, with one capsule taken on day on 0, 7, and 28. The vaccine should be stored in a dry place at +2–8 °C and has a shelf life of 24 months.

5.4.5 Limitations

Limitations of currently available whole-cell killed cholera vaccines are that multiple doses are needed (at least 2) and that efficacy is suboptimal in young children. Of note, due to the inclusion of rCTB the short-term efficacy in young children is relatively higher for *Dukoral*™ as compared to *Shanchol*™, which may be imperative in epidemic outbreak situations. At the other hand, a disadvantage of *Dukoral*™ compared to *Shanchol*™ is that it less protects against the more virulent serogroup O139 that is emerging in the Asian region (rCTB will likely give some protection).

Arguments against the use of oral cholera vaccines in outbreaks include the priority of water provision and cholera treatment measures, low compliance to a multiple-dose regimen due to migration of the target populations, the period required for a two-dose vaccine to generate immunity, the logistic challenges in a setting of inadequate infrastructure and human resources, cold chain requirements, difficulty in transport of bulky vaccine, and clean water requirements for the buffer. In response to these logistical concerns, a vaccination campaign with *Shanchol*™ was initiated in the region of Boffa, in Guinea, in April 2012 (Zorlu 2012): not only is this the first vaccination campaign attempting to control cholera during an active epidemic in Africa, it also aims to collect needed data to get a better understanding into how to optimize the implementation of a vaccination program in response to a cholera outbreak, and to support the development of intervention criteria for the control of cholera in outbreak situations.

5.4.6 Is There a Future for Inactivated Vaccines?

Despite the paradigm that the intestinal mucosa induces immuno-tolerance against nonliving foreign material or commensal flora, oral inactivated cholera vaccines are effective in preventing cholera disease. The existing inactivated vaccines are effective in preventing disease both in children of 2 years old and above in both endemic and immunologically naïve populations. Both *Dukoral*™ and *Shanchol*™ meet the criteria set by the ad hoc Cholera Vaccine Working Group for the performance and characteristics of cholera vaccines for use in public health settings in developing countries, including an efficacy rate of at least 50 % at 2 years of follow-up; a maximum of two doses for initial vaccination, and effectiveness in children from the age of 2 years.

5.4.7 Vaccines in Development

5.4.7.1 Live-Attenuated Vaccines

A live-attenuated oral vaccine may have the advantage over inactivated oral cholera vaccines that it colonizes the intestines, and given in a single dose may provide greater and longer lasting protection. Additionally, more antigens can be preserved or overexpressed, so that a near wild-type-like immune response can be generated. A potential hurdle is that the extensive attenuation of key virulence antigens in order to reduce reactogenicity may also lower the immunogenicity of the vaccine. One live-attenuated oral cholera vaccine has been licensed in the past, CVD 103-HgR: production of this vaccine was suspended in 2004.

CVD-103-HgR (*PXVX-0200*; *Orochol™/Mutacol™*). This was the first successfully developed and only ever-licensed live-attenuated oral cholera vaccine, administered as a single dose. This vaccine—initially developed by the Center for Vaccine Development at the University of Maryland in the 1980s—was derived from Classical O1 Inaba 569B strain, and genetically engineered to express CTB (two gene copies) with most of the active A subunit deleted (deletion of a 550 bp *XbaI*-ClaI fragment internal to the gene encoding the A subunit) to remove toxicity, and harbored a mutation of the haemolysin gene to induce further attenuation (deletion of a 400 bp *HpaI* fragment internal to the haemolysin gene and insertion of a 4.2 kbp fragment from *Shigella flexneri* mercury resistance (*mer*) genes into the *hlyA*s). DNA hybridisation studies on samples taken from the master seed bank and from two production lots grown for up to 17 generations did not identify any rearrangements, hence indicating stability of the genetic modifications (Favre et al. 1996).

In a challenge study involving US volunteers, a single dose of 2–8 × 10^8 live vaccine cells was found to confer 80–100 % protection against challenge with wild-type *Vibrios* at 3 months postimmunization (Tacket et al. 1999). Based on these results, the vaccine was licensed in 1993 as a travelers' vaccine for adults and children ≥2 years in several countries: two formulations were available, one for the travelers market (2–10 × 10^8 CFU) and a higher dosage for endemic populations (2–10 × 10^9 CFU). A single dose was sufficient to induce protective immune responses 8 days postimmunization. Although the vaccine was well tolerated by Chilean infants in a clinical trial setting, the vaccine was never recommended for children under 2 years of age. In the Chilean trial, the vaccine was offered to 312 infants aged 3–17 months old; however, because of its unpleasant taste, only 37 % of the children actually took the vaccine (Lagos et al. 1999).

In a large postlicensure field efficacy trial in Indonesia involving >67,000 subjects, (including children >2 years of age), the vaccine showed very low efficacy (less than 18 % at 1 year follow-up; 2.3 % after 2 years). A very low cholera incidence was put forward as a possible explanation for this disappointing result (Richie et al. 2000). In a retrospective cohort analysis, CVD 103-HgR was found to induce a protective efficacy as high as 79 % (95 % CI 72–84 %) when the vaccine

was used in a mass vaccination campaign to control a cholera outbreak in Micronesia (Calain et al. 2004).

The live-attenuated oral vaccine has a history of safe use with a very low incidence of adverse reactions. There are no reports of toxic or allergic reactions in vaccine recipients. Data from clinical trials have demonstrated that less than 30 % of vaccine recipients excrete detectable numbers of the genetically modified bacteria for a maximum of 7 days (Levine et al. 1988; Simanjuntak et al. 1993). In a clinical trial involving 245 Indonesian children, *V. cholerae* CVD 103-HgR was isolated from the stool culture from only 1 out of 174 family contacts examined (Simanjuntak et al. 1993).

Hundreds of thousands of doses of this oral live-attenuated oral cholera vaccine have been administered worldwide before its production was halted in 2004. PaxVax is currently undertaking efforts to bring the vaccine back to market. The US FDA has accepted an Investigational New Drug application for the product (coded PXVX-0200) and a Phase I clinical trial is underway in the US involving 60 adult volunteers aged 18–15 years (INCT01585181). If successful, PaxVax would be the first to bring a cholera vaccine to the US market.

Peru 15 (*CholeraGarde*™) is a live genetically attenuated nonmotile *V. cholerae* O1 El Tor Inaba strain, originally isolated in Peru in 1991 (Chowdhury et al. 2009). The vaccine strain (developed at Harvard University in the 1990s) harbors deletions in the *ctxa* gene encoding the CTA subunit, the *rtxA* gene encoding the RTX toxin, and the *zot* and *ace* genes. It also lacks flagella, rendering it nonmotile and nonrecombinational.

A single oral dose has been shown to be safe and immunogenic in US volunteers as well as in adults and toddlers in Bangladesh. In a human challenge study, 59 US volunteers were randomly allocated for vaccination (2×10^8 CFU) and challenged with O1 El Tor: 98 % of vaccinees showed at least a fourfold increase in vibriocidal titres and the vaccine was 100 % protective in preventing moderate-to-severe diarrhea. Between 2005 and 2007, age-descending trials were performed in Bangladesh, including subjects as young as 9 months old: 70 % of children aged 9–23 months old developed significant vibriocidal antibody responses to a single dose (Chowdhury et al. 2009). However, an age-related decrease in response was seen in children and infants as compared to adults. To increase responses to CTB, Peru-15 was genetically reengineered to express and secrete higher levels of CTB (by cloning *ctxB* onto a *glnA* balanced-lethal plasmid under the transcriptional control of a strong constitutive promoter), resulting in Peru-15pCTB (Roland et al. 2007; Chowdhury et al. 2009). In mice and rabbits, Peru-15pCTB secreted 30-fold more CTB than the parent strain and elicited 30-fold higher anti-CTB serum IgG responses. A Phase I safety and immunogenicity clinical trial has been completed in the US involving 64 adults aged 18–45 years old healthy adults (NCT00654108).

The Peru-15 blueprint was transferred to O139, resulting in a candidate vaccine that was safe and immunogenic in volunteers and provided 83 % efficacy against experimental O139 challenge 1 month after immunization (Coster et al. 1995).

V. cholerae 638. The live-attenuated oral cholera vaccine candidate *V. cholerae* 638 was constructed in the 1990s in Cuba from O1 El Tor Ogawa strain C7528 by a deletion of the CTXϕ prophage and inactivation of the HA/P gene by insertion of the *Clostridium thermocellum* endoglucanase A gene (*cel*A) into the haemagglutinin/protease *hapA* gene (Benitez et al. 1999). The Finlay Vaccine Institute in Cuba is aiming to further develop this vaccine candidate and have it WHO prequalified for use in cholera-endemic countries. In a Phase I clinical trial, a single dose (1×10^9 CFU) was demonstrated to be immunogenic and protective in an experimental cholera challenge model involving healthy Cuban adults (Garcia et al. 2005). Next, its safety and immunogenicity was confirmed in a placebo-controlled clinical Phase II performed in an endemic cholera setting in Mozambique in which healthy adults received a single dose of 2×10^9 CFU (Garcia et al. 2011).

Others. Other live-attenuated cholera vaccines that are under development include *V. cholerae* IEM 101/108 and VA1.3/VA1.4. IEM 101/108 is being developed in China, and is derived from an O1 El Tor Ogawa strain that naturally lacks the gene for CT and several other virulence factors. VA1.3/VA1.4 is being developed by government-run research laboratories in India and is derived from a nontoxigenic O1 El Tor strain to which the *ctxB* gene was added to produce CTB, but is otherwise devoid of CT.

5.4.8 Conclusions

The currently available oral killed whole-cell cholera vaccines appear to be relatively effective and safe, preventing 50–60 % of cholera episodes during the first 2 years after completion of the primary vaccination schedule. The impact and cost-effectiveness of implementing oral cholera vaccines into the routine vaccination schedules of endemic countries will depend on the prevalence of cholera, the frequency of epidemics, and access to basic services providing rapid rehydration therapy. Whole-cell/rCTB cholera vaccines are beneficial for travelers to zones where cholera and travelers' diarrhea are a risk. Live-attenuated vaccines that induce protective immunity after a single dose may eventually be available and help to protect people living in low-income countries at risk of cholera outbreaks.

5.5 Typhoid Fever Vaccine

5.5.1 Typhoid Fever: Overview of Bacteriology and Pathology

Enteric fever is an acute feverish illness caused by ingestion of food or water contaminated with *Salmonella enterica* serovar Typhi (typhoid fever) or Paratyphi A, B, or less frequently, C (paratyphoid fever). *S. enterica* are rod-shaped facultative-anaerobic

Gram-negative bacteria that are commonly found in raw meat, chicken, and egg shells. The global annual incidence of enteric fever is around 21 million cases, including 200,000–600,000 deaths, and mainly involves typhoid fever (an estimated 17 million cases per year) (Crump et al. 2004). Incidence is the highest in Asia and Northern Africa.

Central to the pathogenicity of serovar Typhi is its capacity to invade intestinal epithelial cells. When left untreated, infections can become life-threatening due to perforation of the wall of the small intestine, intestinal bleeding, dissemination of the infection into the blood stream and potentially the brain, and inflammation of other organs such as the gallbladder and the heart. High-risk groups include travelers to endemic areas, and infants, children and young adults residing in endemic areas. Disease peaks in younger age-groups when endemicity is higher (Crump et al. 2004; Lin et al. 2000) (Fig. 5.4). A sero-epidemiological study in Nepal has indeed provided evidence that *S. typhi*-specific bactericidal antibody titres increase with age in an endemic setting (Pulickal et al. 2009): this naturally acquired immunity may involve antibody responses against immunodominant antigens such as the somatic O antigen, the Vi capsular polysaccharide, iron-regulated outer membrane proteins (OMP), and porins. Although a correlate of protection has not yet been identified, serum IgG antibodies (seroconversion) and gut-derived IgA antibody-secreting cells are putatively immunological surrogates of protection (Pasetti et al. 2011), as well as that cell-mediated immunity is assumed to play a role. Typhoid vaccine development has been hindered by the facts that *Salmonella* enteric serovar Typhi infections are restricted to humans and that no serological correlate of protection has yet been identified. Human challenge models in which volunteers deliberately ingested wild-type *Salmonella* serovar *Typhi* Quailes to study vaccine efficacy and pathogenesis were done in the 1960–1970s. The human challenge model may undergo a revival due to the availability of the highly sensitive TSB-bile blood culture PCR system that allows faster (less than 8 h) and more

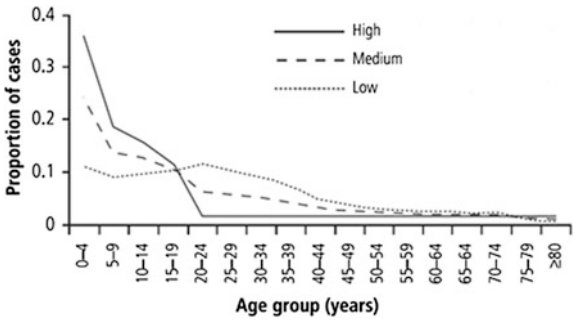


Fig. 5.4 Age-related distribution of typhoid fever depending on endemicity. Endemicity was defined based on incidence per year with high endemicity >100 cases/100,000; medium endemicity 10–100/100,000; and low endemicity <10/100,000. Reproduced with permission from the World Health Organization (Crump et al. 2004)

sensitive detection of *S. typhi* infection and hence quicker intervention options as compared to time-consuming conventional blood culture and PCR methods (Zhou and Pollard 2010).

5.5.2 Vaccines Licensed and in Use

The first *S. typhi* vaccines developed were heat-killed and acetone-killed whole-cell vaccines. Due to their high reactogenicity, including local inflammation, pain, systemic fever, malaise, and disease-like symptoms, these vaccines are no longer recommended for use (Garmory et al. 2002). Currently, there are two typhoid vaccines on the market: a multidose oral live-attenuated vaccine (Ty21a), and a parental single-dose Vi capsular polysaccharide vaccine.

5.5.3 Live-AttenuatedVaccines

Ty21a was developed in the 1970s and currently still is the only licensed live-attenuated typhoid vaccine available (now produced and marketed as *Vivotif*™. Ty21a is derived from a wild-type *S. typhi* Ty2 strain and has been attenuated through chemical mutagenesis. The attenuating genetic alternations are to a large extent still uncharacterized. One of key mutations is the absence of a functional galactose-epimerase (*galE*) gene (Kopecko et al. 2009). Since galactose is incorporated into the core oligosaccharide and O-polysaccharide (O-PS), the absence of *galE* leads to the formation of rough LPS during growth in the absence of exogenous galactose. Since O-PS is the main antigenic determinant on the cell surface, Ty21a is supplied with a limiting source of exogenous galactose during the production process to allow minor, self-limiting replication and expression of O antigens via a non-*galE*-dependent pathway. Following oral vaccine administration, the presence of low concentrations of galactose in the intestines facilitates the production of sufficient amounts of O-LPS and brief in vivo multiplication of the vaccine strain and induction of a protective local immune response. Ty21a is believed to remain locally in the intestine and has never been detected systemically or in stool after the usual dose. In addition to the multiple mutations in the galactose metabolism, synthesis of the Vi polysaccharide capsule is mutated, leading to a further attenuation. Mutations have been stable for Ty21a master—and working seed lots (MSL and WSL) produced since 1980 until at least 2005 (Kopecko et al. 2009).

Given the close homology in many key antigenic determinants between *S. enterica* serovars Typhi and Paratyphi A and B (among which are the outer membrane proteins OMP C and OMP F, and the O12 LPS, although the latter is not expressed by all *S. typhi*) (Liu et al. 1991; Nikaido 2003), Ty21a has the potential to elicit cross-protection against paratyphoid fever (Levine et al. 2007). Two large field trials in Chile found that Ty21a conferred moderate protection (49 %, CI is

8–73 %) against paratyphoid B infections, but not paratyphoid A—which was explained by the low number of paratyphoid A cases (only three cases in the vaccine group and four cases in the placebo group) (Levine et al. 2007). Recently, Ty21a was shown to induce cross-reactive LPS-specific humoral immune responses (including antibody-secreting cells, memory B-cells and serum titres) in vaccinated adult volunteers, with cross-reactive responses being higher for *S. paratyphi* B than *S. paratyphi* A (Wahid et al. 2012). The same was found using whole bacteria as antigen sources (Pakkanen et al. 2012). Up to today, it remains unclear whether Ty21a has any potential to induce cross-protection against *Paratyphi* A, or whether this is restricted to *Paratyphi* B only.

The immunization regimen for Ty21a is 3 or 4 doses (varying per country), given 2 days apart, with one dose containing at least 2×10^9 viable organisms in lyophilised form. Three oral doses of Ty21a administered in enteric-coated capsules have been shown to confer 67 % protection against typhoid fever for 3 years post-stimmunization, with a sustained (62 %) protective efficacy over a period of 7 years (Levine et al. 1999).Targeted vaccination strategies with Ty21a is widely used and accepted; however, more often in mass vaccination programs, and not as routine immunization.

5.5.4 Limitations

Ty21a is used mainly as a travelers' vaccine for the US, EU and other high-income markets. Ty21a is infrequently used in endemic settings for several reasons. First of all, the vaccine possesses low thermal stability, having a shelf life of 18 months when stored between 2 and 8 °C, and 14 days only at 25 °C whilst quickly losing viability when exposed to elevated temperatures (Corbel 1996). Increasing the vaccine's heat stability would be a considerable improvement. One such possibility may be foam drying as demonstrated by Ohtake et al. (2011): a foam-dried live-attenuated *S. typhi* Ty21a vaccine was stable for approximately 12 weeks at 37 °C (i.e., the time required for the vaccine to decrease in potency by $^1\log_{10}$ CFU), and showed no loss in titre at 4 and 25 °C following storage for the same duration. The foam-dried vaccine induced higher immune responses (serum LPS-IgG titres) in mice compared to the commercially available vaccine. As proposed by the authors, the enhanced heat stability of the Ty21a oral vaccine could mitigate risks of vaccine potency loss during long-term storage, shipping, and delivery to geographical areas with warmer climates.

A second limitation is that to achieve immune protection, 3–4 doses of Ty21a need to be taken at 2 day intervals. This is associated with a risk of noncompliance in settings where intake is not controlled. Vaccines requiring only one dose to reach comparable long-lasting immunogenicity would be preferable.

Thirdly, Ty21a is currently only available in enteric-coated capsules that are recommended for use in adults and children over 5 years of age. A liquid formulation that would allow immunization of younger children (≥2 years old) is licensed

and has been marketed; however, it is currently not produced, despite the need to immunize children younger than 5 years of age as they particularly are at high risk in more endemic settings (Fig. 5.4).

Finally, the price of the vaccine is prohibitive for developing countries that have restricted national health budgets.

5.5.5 *Is There a Future for "Ty21a", the Only Live-Attenuated* Salmonella typhi *Vaccine?*

Ever since its launch in the early 1980s, Ty21a has been a successful vaccine with an extremely good safety profile and inducing protection for at least 5–7 years against *S. typhi* infections. The only other *S. typhi* vaccine that is currently on the market is a parenteral single-dose Vi capsular polysaccharide vaccine. Nonetheless, there are many other typhoid vaccines in development, including a Vi capsular conjugate vaccine that is likely to show superior immunogenicity as compared to the plain polysaccharide vaccine, and several genetically attenuated vaccines (discussed below). The future will determine how well these new vaccine candidates compare to the long-standing Ty21a live-attenuated vaccine.

S. typhi Ty21a has also been tried and used as a vaccine delivery vector for several heterologous targets, including other bacterial targets such as *Shigella sonnei* and *dysenteriae* antigens (Black et al. 1987; Formal et al. 1981), anthrax antigens (Osorio et al. 2009), and more recently also anticancer siRNAs (Xu et al. 2009). The future of Ty21a as a vaccine vector may hence be promising.

5.5.6 *Vaccines in Development*

5.5.6.1 Live-Attenuated Vaccines

With the modern era of molecular biology, new genetically attenuated *S. typhi* strains that harbor precise attenuating mutations have been engineered. Genetic attenuation initially focused on serovar Typhi derivatives with either single or double *aro* mutations affecting the purine and aromatic biosynthetic pathways, rendering the vaccine strain nutritionally dependent on substrates that are unavailable in mammalian tissues. This prevents the bacterium from proliferating in mammalian cells whilst growing long enough to stimulate immune responses. Although these *aro* mutants were attenuated and highly immunogenic, human volunteer studies demonstrated that immunization with these mutant strains gave rise to bacteraemia in some cases. This fuelled a search for additional attenuating mutations which, when introduced into an *aro* mutant, would offer a vaccine with retained immunogenicity that did not cause bacteraemia. Genetically, live-attenuated

Table 5.4 Main genetically live-attenuated typhoid vaccines in development

Vaccine	Strain	Genetic attenuation	Product profile	Trials
CVD909	Ty2[a]	*aroC/aroD*-deletion mutation *htrA*	Oral	Phase I safety study in healthy adults completed
		P_{tviA} replaced with P_{tac},		Prime-boost study (CVD909 prime + polysaccharide Vi booster) completed
M01ZH09/ Typhella	Ty2[a]	mutations *aroC* and *ssaV*	Oral (drinkable) ≥2 years of age	Phase II immunogenicity, safety and tolerability study in healthy adults completed
			Endemic and travelers	Phase II immunogenicity, safety and tolerability study in children aged 5–14 years living in typhoid endemic area in Vietnam completed
				Phase II human typhoid infection study ongoing
Ty800	Ty2[a]	*phoP/phoQ* mutations		Phase I/II dose-ranging safety and immunogenicity study in healthy adults completed

[a] Ty2 is parent strain of Ty21a

strains that are currently in development (Table 5.4) aim at equal tolerability as Ty21a, superior immunogenicity and protection, and the requirement of just a single oral dose.

CVD 909 (Center for Vaccine Development, University of Maryland, Baltimore, U.S.) harbors three genetically attenuating mutations (Tacket and Levine 2007): 1. Deletion in *aroC/aroD*, as mentioned earlier; 2. A mutation in the *htrA* gene encoding for a heat shock protein, which further impairs the ability of the vaccine strain to survive and replicate in host tissues; and 3. Replacement of the highly regulated promoter P_{tviA} with the constitutive promoter P_{tac} to induce continuous expression of Vi (during infections with wild-type *S. typhi* Vi expression is turned off), and increase vaccine immunogenicity and protective efficacy. Of note, in a heterologous prime-boost study, mucosal priming with CVD 909 was found to enhance immune responses induced by subsequent parenteral administration of the Vi polysaccharide vaccine, as characterized by persistent Vi-specific IgA B memory cells (Wahid et al. 2011).

M01ZH09/Typhella (Emergent BioSolutions, Rockville, U.S.) (Jain 2009; Tran et al. 2010) is a drinkable, single-dose live-attenuated typhoid vaccine based on a proprietary *spi*-VEC™ platform, with two independently attenuating deletions: one, again, in the *aroC* gene; and an additional one in *ssaV*, a gene encoding for a structural component of the *Salmonella* pathogenicity island-2, SPI-2, a type III secretion system. Consequently, the ability of the vaccine strain to survive in the bloodstream is impaired, hence eliminating the risk of postimmunization bacteraemia (Hindle et al.

2002). M01ZH09 has been demonstrated to be safe and immunogenic in studies involving the UK and the US healthy adult volunteers, and healthy 5–14 years old Vietnamese children living in an endemic setting (oral single dose) (Tran et al. 2010). In the last trial, 51 % of vaccine recipients shed the *S. typhi* vaccine strain in stools after vaccination: one subject excreted the vaccine strain on day 3, but no shedding was observed on day 4 and beyond. In the study with UK and US adult volunteers, shedding of *S. typhi* in stools was reported for slightly longer duration.

Ty800 (AVANT Immunotherapeutics) was developed by deletion of the *phoP/phoQ* genes in the *S. typhi* strain Ty2. PhoP/Q is required for survival in macrophages, and mutations in *phoP/Q* result in a marked decrease in virulence (Charles et al. 2009). Initially, these genetic deletions were constructed in the aromatic amino acid auxotrophic vaccine strain *S. typhi* 514Ty (Ty2 Δ*aroA hisG46*) (Ty445); however, given that very large doses of Ty445 were well tolerated, Ty800 (not including the Δ*aroA hisG46* mutations) was developed with the aim of enhancing immunogenicity (Hohmann et al. 1996).

S. paratyphi A. Several genetically attenuated *S. paratyphi* A vaccine strains have been developed, including the candidate vaccine CVD 1902 that is currently tested in a Phase I clinical trial. This genetically live-attenuated vaccine strain harbors deletions in the *guaBA* and *clpX* genes, introduced in wild-type *S. paratyphi* strain ATCC 9150. A deletion in the *guaBA* locus impedes virulence by inhibiting guanine nucleotide biosynthesis (Wang et al. 2001), whereas a deletion in *clpX* (encoding ATPase) results in flagella overexpression and hence is aiming at increased immunogenicity (Tomovasu et al. 2002). This is the first live-attenuated *S. paratyphi* A vaccine tested in humans.

5.5.7 Invasive Nontyphoidal Salmonella (INTS)

While nontyphoidal *Salmonella* (NTS) has long been recognized as a cause of self-limiting gastroenteritis, NTS serovars Typhimurium and Enteritidis are emerging as important causes of invasive bloodstream infection in sub-Saharan Africa, in particular in young children suffering from malaria and malnutrition, and adults with HIV. Full genome sequencing of an invasive prototype *S. typhimurium* strain from Malawi revealed that the strain manifested considerable genomic degradation, including complete loss of some genes and the presence of multiple pseudogenes, some of which are also found in *S. typhi* or *S. paratyphi A*.

Several live-attenuated *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis strains have been developed and tested as vaccine candidates preclinically (Simon et al. 2011; Tennant et al. 2011). Using two invasive NTS (iNTS) strains isolated from the blood of toddlers in Mali (wild-type strains *S. typhimurium* I77 and *S. enteritidis* R11), prototype live-attenuated vaccine strains CVD 1921 and CVD 1941 were constructed by deleting *guaBA*, encoding guanine biosynthesis, and *clpP*, encoding a master protease regulator. An additional deletion in *fliD* yielded strains CVD 1923 and CVD 1943, respectively, which export flagellin

monomers. Oral immunization with CVD 1921 or CVD 1923 protected mice against lethal challenge with wild-type iNTS *Typhimurium* I77. Immunization with CVD 1941, but not CVD 1943, protected mice against lethal challenge with *S. enteritidis* R11. Moreover, vaccine-induced immune responses, including serum anti-LPS and anti-flagellum IgG antibodies gradually increased during the immunization course. Having demonstrated preclinical proof of concept, live-attenuated *S. typhimurium* and *S. enteritidis* candidate vaccine strains carrying mutations in *guaBA* and *clpPX* are expected to move to Phase I human trials.

5.5.8 Conclusions

Ty21a is an effective live-attenuated vaccine offering protection against typhoid fever, and some cross-protection against paratyphoid B. The vaccine is suitable for travelers to endemic countries, but its physical characteristics and dose requirements make it less suitable for high-risk endemic settings.

Several genetically attenuated *S. typhi* vaccine strains harboring precise attenuating mutations are currently undergoing evaluation in clinical trials, and may in the future offer live-attenuated typhoid vaccines with higher stability and a one-dose only immunization regimen: however, at the time of writing no genetically live-attenuated bacterial vaccine has yet received regulatory approval. In addition, two *S. typhi* capsular polysaccharide Vi conjugate vaccines are under development: Vi-rEPA (Vi bound to recombinant exoprotein A of *Pseudomonas aeruginosa*) and Vi-CRM_{197} that the Novartis Vaccines Institute for Global Health (NVGH) with funding from the Welcome Trust may try developing into a bivalent combining it with the *S. paratyphi* A O-specific polysaccharide conjugated to CRM_{197} (O:2-CRM_{197}). Conjugate approaches are also being taken independent of each other by the NVGH and University of Maryland Center for Vaccine Development (with funding from Wellcome Trust) to develop an O antigen glycoconjugate vaccine against iNTS.

5.6 Conclusion

The techniques for producing whole-cell and live attenuated vaccines date back more than a century. Since that time, highly sophisticated molecular methods for identifying, reproducing, and manufacturing specific antigens targeting bacterial diseases have been developed. Nevertheless, the production of live-attenuated and whole-cell vaccine continues, with their administration to many thousands or millions of individuals each year. The theoretical advantages of administering live-attenuated vaccines, which includes induction of a broader and longer lasting immune response, the need for fewer doses, and the possibility to immunize very

young children, means that investigation of live-attenuated vaccine options to prevent bacterial diseases continues unabated. New whole-cell vaccines are also being investigated as part of the fight against tuberculosis. However, the continued success of live-attenuated and whole-cell vaccines will depend upon the parallel development of methods that reliably assess the quality, potency, immunogenicity, and efficacy of these vaccines.

References

Aaby P, Benn CS (2012) Saving lives by training innate immunity with bacille Calmette-Guerin vaccine. Proc Natl Acad Sci USA 109(43):17317–17318. doi:10.1073/pnas.1215761109

Alam M, Sultana M, Nair GB, Siddique AK, Hasan NA, Sack RB, Sack DA, Ahmed KU, Sadique A, Watanabe H, Grim CJ, Huq A, Colwell RR (2007) Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission. Proc Natl Acad Sci USA 104(45):17801–17806. doi:10.1073/pnas.0705599104

Alrabiaah AA, Alsubaie SS, Bukhari EI, Gad A, Alzamel FA (2012) Outbreak of bacille Calmette-Guerin-related lymphadenitis in Saudi children at a university hospital after a change in the strain of vaccine. Ann Saudi Med 32(1):4–8

Aporta A, Arbues A, Aguilo JI, Monzon M, Badiola JJ, de Martino A, Ferrer N, Marinova D, Anel A, Martin C, Pardo J (2012) Attenuated *Mycobacterium tuberculosis* SO_2 vaccine candidate is unable to induce cell death. PLoS ONE 7(9):e45213. doi:10.1371/journal.pone.0045213

Ausiello CM, Urbani F, la Sala A, Lande R, Cassone A (1997) Vaccine- and antigen-dependent type 1 and type 2 cytokine induction after primary vaccination of infants with whole-cell or acellular pertussis vaccines. Infect Immun 65(6):2168–2174

Benitez JA, Garcia L, Silva A, Garcia H, Fando R, Cedre B, Perez A, Campos J, Rodriguez BL, Perez JL, Valmaseda T, Perez O, Ramirez M, Ledon T, Jidy MD, Lastre M, Bravo L, Sierra G (1999) Preliminary assessment of the safety and immunogenicity of a new CTXPhi-negative, hemagglutinin/protease-defective El Tor strain as a cholera vaccine candidate. Infect Immun 67 (2):539–545

Black RE, Levine MM, Clements ML, Losonsky GA, Herrington D, Berman S, Formal SB (1987) Prevention of shigellosis by a *Salmonella typhi–Shigella sonnei* bivalent vaccine. J Infect Dis 155(6):1260–1265

Bordet J, Gengou U (1906) Le microbe de la coqueluche. Ann Inst Pasteur 20:48–68

Burgess M, Forrest J (1996) Pertussis and the acellular vaccines. Commun Dis Intell 20:192–193

Burl S, Adetifa UJ, Cox M, Touray E, Ota MO, Marchant A, Whittle H, McShane H, Rowland-Jones SL, Flanagan KL (2010) Delaying bacillus Calmette-Guerin vaccination from birth to 4 1/2 months of age reduces postvaccination Th1 and IL-17 responses but leads to comparable mycobacterial responses at 9 months of age. J Immunol 185(4):2620–2628. doi:10.4049/jimmunol.1000552

Calain P, Chaine JP, Johnson E, Hawley ML, O'Leary MJ, Oshitani H, Chaignat CL (2004) Can oral cholera vaccination play a role in controlling a cholera outbreak? Vaccine 22 (19):2444–2451

Campbell H, Amirthalingam G, Andrews N, Fry NK, George RC, Harrison TG, Miller E (2012) Accelerating control of pertussis in England and Wales. Emerg Infect Dis 18(1):38–47. doi:10.3201/eid1801.110784

Canaday DH, Wilkinson RJ, Li Q, Harding CV, Silver RF, Boom WH (2001) CD4(+) and CD8(+) T cells kill intracellular *Mycobacterium tuberculosis* by a perforin and Fas/Fas ligand-independent mechanism. J Immunol 167(5):2734–2742

Capinos Scherer CF, Endsley JJ, de Aguiar JB, Jacobs WR Jr, Larsen MH, Palmer MV, Nonnecke BJ, Ray Waters W, Mark Estes D (2009) Evaluation of granulysin and perforin as candidate biomarkers for protection following vaccination with *Mycobacterium bovis* BCG or *M. bovis*DeltaRD1. Transbound Emerg Dis 56(6–7):228–239. doi:10.1111/j.1865-1682.2008.01058.x

Cardona PJ, Asensio JG, Arbues A, Otal I, Lafoz C, Gil O, Caceres N, Ausina V, Gicquel B, Martin C (2009) Extended safety studies of the attenuated live tuberculosis vaccine SO_2 based on phoP mutant. Vaccine 27(18):2499–2505. doi:10.1016/j.vaccine.2009.02.060

Centers for Disease Control and Prevention (2002) Pertussis–United States, 1997–2000. MMWR Morb Mortal Wkly Rep 51(4):73–76

Chackerian AA, Perera TV, Behar SM (2001) Gamma interferon-producing CD4+ T lymphocytes in the lung correlate with resistance to infection with *Mycobacterium tuberculosis*. Infect Immun 69(4):2666–2674. doi:10.1128/IAI.69.4.2666-2674.2001

Charles RC, Harris JB, Chase MR, Lebrun LM, Sheikh A, Larocque RC, Logvinenko T, Rollins SM, Tarique A, Hohmann EL, Rosenberg I, Krastins B, Sarracino DA, Qadri F, Calderwood SB, Ryan ET (2009) Comparative proteomic analysis of the PhoP regulon in *Salmonella enterica* serovar Typhi versus Typhimurium. PLoS ONE 4(9):e6994

Cherry JD (1996) Historical review of pertussis and the classical vaccine. J Infect Dis 174(Suppl 3):S259–S263

Chowdhury MI, Sheikh A, Qadri F (2009) Development of Peru-15 (CholeraGarde), a live-attenuated oral cholera vaccine: 1991–2009. Expert Rev Vaccines 8(12):1643–1652

Clemens J, Shin S, Sur D, Nair GB, Holmgren J (2011) New-generation vaccines against cholera. Nat Rev Gastroenterol Hepatol 8(12):701–710

Clemens JD, Harris JR, Sack DA, Chakraborty J, Ahmed F, Stanton BF, Khan MU, Kay BA, Huda N, Khan MR et al (1988) Field trial of oral cholera vaccines in Bangladesh: results of one year of follow-up. J Infect Dis 158(1):60–69

Clemens JD, Sack DA, Harris JR, Van Loon F, Chakraborty J, Ahmed F, Rao MR, Khan MR, Yunus M, Huda N et al (1990) Field trial of oral cholera vaccines in Bangladesh: results from three-year follow-up. Lancet 335(8684):270–273

Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM (1993) Disseminated tuberculosis in interferon gamma gene-disrupted mice. J Exp Med 178(6):2243–2247

Corbel MJ (1996) Reasons for instability of bacterial vaccines. Dev Biol Stand 87:113–124

Corbel MJ, Kreeftenberg JG, Knezevic I (2004) WHO Working Group on the standardisation and control of pertussis vaccines-report of a meeting held on 6–7 May 2003, Ferney Voltaire, France. Vaccine 22(3–4):293–300

Corbel MJ, Xing DK (2004) Toxicity and potency evaluation of pertussis vaccines. Expert Rev Vaccines 3(1):89–101. doi:10.1586/14760584.3.4.S89

Cornford-Nairns R, Daggard G, Mukkur T (2012) Construction and preliminary immunobiological characterization of a novel, non-reverting, intranasal live attenuated whooping cough vaccine candidate. J Microbiol Biotechnol 22(6):856–865

Cortese MM, Baughman AL, Zhang R, Srivastava PU, Wallace GS (2008) Pertussis hospitalizations among infants in the United States, 1993 to 2004. Pediatrics 121(3):484–492. doi:10.1542/peds.2007-1393

Coster TS, Killeen KP, Waldor MK, Beattie DT, Spriggs DR, Kenner JR, Trofa A, Sadoff JC, Mekalanos JJ, Taylor DN (1995) Safety, immunogenicity, and efficacy of live attenuated *Vibrio cholerae* O139 vaccine prototype. Lancet 345(8955):949–952

Crowcroft NS, Booy R, Harrison T, Spicer L, Britto J, Mok Q, Heath P, Murdoch I, Zambon M, George R, Miller E (2003) Severe and unrecognised: pertussis in UK infants. Arch Dis Child 88(9):802–806

Crump JA, Luby SP, Mintz ED (2004) The global burden of typhoid fever. Bull World Health Organ 82(5):346–353

Davisson EO, Powell HM, Macfarlane JO, Hodgson R, Stone RL, Culbertson CG (1956) The preservation of poliomyelitis vaccine with stabilized merthiolate. J Lab Clin Med 47(1):8–19

de Greeff SC, de Melker HE, Westerhof A, Schellekens JF, Mooi FR, van Boven M (2012) Estimation of household transmission rates of pertussis and the effect of cocooning vaccination strategies on infant pertussis. Epidemiology 23(6):852–860. doi:10.1097/EDE.0b013e31826c2b9e

de Melker HE, Conyn-van Spaendonck MA, Rumke HC, van Wijngaarden JK, Mooi FR, Schellekens JF (1997) Pertussis in the Netherlands: an outbreak despite high levels of immunization with whole-cell vaccine. Emerg Infect Dis 3(2):175–178. doi:10.3201/eid0302.970211

De Serres G, Shadmani R, Duval B, Boulianne N, Dery P, Douville Fradet M, Rochette L, Halperin SA (2000) Morbidity of pertussis in adolescents and adults. J Infect Dis 182 (1):174–179. doi:10.1086/315648

Denoel P, Godfroid F, Guiso N, Hallander H, Poolman J (2005) Comparison of acellular pertussis vaccines-induced immunity against infection due to *Bordetella pertussis* variant isolates in a mouse model. Vaccine 23(46–47):5333–5341. doi:10.1016/j.vaccine.2005.06.021

Diel R, Goletti D, Ferrara G, Bothamley G, Cirillo D, Kampmann B, Lange C, Losi M, Markova R, Migliori GB, Nienhaus A, Ruhwald M, Wagner D, Zellweger JP, Huitric E, Sandgren A, Manissero D (2011) Interferon-gamma release assays for the diagnosis of latent *Mycobacterium tuberculosis* infection: a systematic review and meta-analysis. Eur Respir J 37(1):88–99. doi:10.1183/09031936.00115110

Donoghue HD, Lee OY, Minnikin DE, Besra GS, Taylor JH, Spigelman M (2010) Tuberculosis in Dr Granville's mummy: a molecular re-examination of the earliest known Egyptian mummy to be scientifically examined and given a medical diagnosis. Proc Biol Sci 277(1678):51–56. doi:10.1098/rspb.2009.1484

Edwards KM (2005) Overview of pertussis: focus on epidemiology, sources of infection, and long term protection after infant vaccination. Pediatr Infect Dis J 24(6 Suppl):S104–S108

Edwards KM, Decker MD, Halsey NA, Koblin BA, Townsend T, Auerbach B, Karzon DT (1991) Differences in antibody response to whole-cell pertussis vaccines. Pediatrics 88(5):1019–1023

Ernst WA, Thoma-Uszynski S, Teitelbaum R, Ko C, Hanson DA, Clayberger C, Krensky AM, Leippe M, Bloom BR, Ganz T, Modlin RL (2000) Granulysin, a T cell product, kills bacteria by altering membrane permeability. J Immunol 165(12):7102–7108

Favre D, Struck MM, Cryz SJ Jr, Viret JF (1996) Further molecular characterization and stability of the live oral attenuated cholera vaccine strain CVD103-HgR. Vaccine 14(6):526–531

Feunou PF, Bertout J, Locht C (2010a) T- and B-cell-mediated protection induced by novel, live attenuated pertussis vaccine in mice. Cross protection against parapertussis. PLoS ONE 5(4): e10178 doi:10.1371/journal.pone.0010178

Feunou PF, Kammoun H, Debrie AS, Mielcarek N, Locht C (2010b) Long-term immunity against pertussis induced by a single nasal administration of live attenuated *B. pertussis* BPZE1. Vaccine 28(43):7047–7053. doi:10.1016/j.vaccine.2010.08.017

Fine PE (1995) Variation in protection by BCG: implications of and for heterologous immunity. Lancet 346(8986):1339–1345

Fine PE, Clarkson JA (1987) Reflections on the efficacy of pertussis vaccines. Rev Infect Dis 9 (5):866–883

Fine PE, Ponnighaus JM, Maine NP (1986) The relationship between delayed type hypersensitivity and protective immunity induced by mycobacterial vaccines in man. Lepr Rev 57 (Suppl 2):275–283

Fine PE, Sterne JA, Ponnighaus JM, Rees RJ (1994) Delayed-type hypersensitivity, mycobacterial vaccines and protective immunity. Lancet 344(8932):1245–1249

Fleming PJ, Blair PS, Platt MW, Tripp J, Smith IJ, Golding J (2001) The UK accelerated immunisation programme and sudden unexpected death in infancy: case-control study. BMJ 322(7290):822

Formal SB, Baron LS, Kopecko DJ, Washington O, Powell C, Life CA (1981) Construction of a potential bivalent vaccine strain: introduction of *Shigella sonnet* form I antigen genes into the galE *Salmonella typhi* Ty21a typhoid vaccine strain. Infect Immun 34(3):746–750

Galazka A (1993) Pertussis. In The immunological basis for immunization. WHO/EPI/GEN/93.14:1–20

Gale JL, Thapa PB, Wassilak SG, Bobo JK, Mendelman PM, Foy HM (1994) Risk of serious acute neurological illness after immunization with diphtheria-tetanus-pertussis vaccine. A population-based case-control study. JAMA 271(1):37–41

Garcia HM, Thompson R, Valera R, Fando R, Fumane J, Jani I, Mirabal M, Armesto MI, Songane M, Luis S, Nzualo AM, Celeste J, Viegas S, Gudo ES, Melembe A, Bila D, Cema C, Mabumo C, Garcia L, Cedre B, Ano G, Martinez JC, Mandarioti A, Lugones J, Gonzalez D, Baro M, Hernandez J, Talavera A, Solis RL, Sierra G, Barbera R, Dominguez F, Gutierrez C, Campa C, Garrido I, Menendez J (2011) A single dose of live-attenuated 638 *Vibrio cholerae* oral vaccine is safe and immunogenic in adult volunteers in Mozambique. VacciMonitor 20(3):1–8

Garcia L, Jidy MD, Garcia H, Rodriguez BL, Fernandez R, Ano G, Cedre B, Valmaseda T, Suzarte E, Ramirez M, Pino Y, Campos J, Menendez J, Valera R, Gonzalez D, Gonzalez I, Perez O, Serrano T, Lastre M, Miralles F, Del Campo J, Maestre JL, Perez JL, Talavera A, Perez A, Marrero K, Ledon T, Fando R (2005) The vaccine candidate *Vibrio cholerae* 638 is protective against cholera in healthy volunteers. Infect Immun 73(5):3018–3024

Garmory HS, Brown KA, Titball RW (2002) Salmonella vaccines for use in humans: present and future perspectives. FEMS Microbiol Rev 26(4):339–353

Greco D, Salmaso S, Mastrantonio P, Giuliano M, Tozzi AE, Anemona A, Ciofi degli Atti ML, Giammanco A, Panei P, Blackwelder WC, Klein DL, Wassilak SG (1996) A controlled trial of two acellular vaccines and one whole-cell vaccine against pertussis. Progetto Pertosse Working Group. N Engl J Med 334(6):341–348. doi:10.1056/NEJM199602083340601

Griffin MR, Ray WA, Mortimer EA, Fenichel GM, Schaffner W (1990) Risk of seizures and encephalopathy after immunization with the diphtheria-tetanus-pertussis vaccine. JAMA 263 (12):1641–1645

Grimprel E, Begue P, Anjak I, Njamkepo E, Francois P, Guiso N (1996) Long-term human serum antibody responses after immunization with whole-cell pertussis vaccine in France. Clin Diagn Lab Immunol 3(1):93–97

Grode L, Seiler P, Baumann S, Hess J, Brinkmann V, Nasser Eddine A, Mann P, Goosmann C, Bandermann S, Smith D, Bancroft GJ, Reyrat JM, van Soolingen D, Raupach B, Kaufmann SH (2005) Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guerin mutants that secrete listeriolysin. J Clin Invest 115(9):2472–2479. doi:10.1172/JCI24617

Guiso N, Capiau C, Carletti G, Poolman J, Hauser P (1999) Intranasal murine model of *Bordetella pertussis* infection. I. Prediction of protection in human infants by acellular vaccines. Vaccine 17(19):2366–2376

Gustafsson L, Hallander HO, Olin P, Reizenstein E, Storsaeter J (1996) A controlled trial of a two-component acellular, a five-component acellular, and a whole-cell pertussis vaccine. N Engl J Med 334(6):349–355. doi:10.1056/NEJM199602083340602

Hallander H, Nilsson L, Gustafsson L (2011) Is adolescent pertussis vaccination preferable to natural booster infections? Expert reviews of clinical pharmacology

Harris JB, LaRocque RC, Qadri F, Ryan ET, Calderwood SB (2012) Cholera. Lancet 379 (9835):2466–2476. doi:10.1016/S0140-6736(12)60436-X

Health Council of the Netherlands (2004) Vaccination against pertussis (Vaccinatie tegen kinkhoest). The Hague

Heininger U (2010) Update on pertussis in children. Expert Rev Anti Infect Ther 8(2):163–173

Hindle Z, Chatfield SN, Phillimore J, Bentley ML, Johnson J (2002) Characterization of *Salmonella enterica* derivatives harboring defined aroC and *Salmonella pathogenicity* island 2 type III secretion system (ssaV) mutations by immunization of healthy volunteers. Vaccine 24:116–123

Ho SY, Chua SQ, Foo DG, Locht C, Chow VT, Poh CL, Alonso S (2008) Highly attenuated *Bordetella pertussis* strain BPZE1 as a potential live vehicle for delivery of heterologous vaccine candidates. Infect Immun 76(1):111–119. doi:10.1128/IAI.00795-07

Hoffman HJ, Hunter JC, Damus K, Pakter J, Peterson DR, van Belle G, Hasselmeyer EG (1987) Diphtheria-tetanus-pertussis immunization and sudden infant death: results of the National Institute of Child Health and Human Development Cooperative Epidemiological Study of Sudden Infant Death Syndrome risk factors. Pediatrics 79(4):598–611

Hoft DF, Blazevic A, Abate G, Hanekom WA, Kaplan G, Soler JH, Weichold F, Geiter L, Sadoff JC, Horwitz MA (2008) A new recombinant bacille Calmette-Guerin vaccine safely induces significantly enhanced tuberculosis-specific immunity in human volunteers. J Infect Dis 198 (10):1491–1501. doi:10.1086/592450

Hohmann EL, Oletta CA, Killeen KP, Miller SI (1996) phoP/phoQ-deleted *Salmonella typhi* (Ty800) is a safe and immunogenic single-dose typhoid fever vaccine in volunteers. J Infect Dis 173(6):1408–1414

Howson CP, Fineberg HV (1992) Adverse events following pertussis and rubella vaccines. Summary of a report of the Institute of Medicine. JAMA 267(3):392–396

iDrugInfo.com (2000–2012) Pentacoq. In: Drugs information. http://www.idruginfo.com/drugs-p/pen/pentacoq.html

Jain SK (2009) M-01ZH09, an oral live attenuated *Salmonella enterica* serovar Typhi vaccine for the prevention of typhoid fever. Curr Opin Mol Ther 11(5):565–571

Jefferson T, Rudin M, DiPietrantonj C (2003) Systematic review of the effects of pertussis vaccines in children. Vaccine 21(17–18):2003–2014

Kammoun H, Feunou PF, Foligne B, Debrie AS, Raze D, Mielcarek N, Locht C (2012) Dual mechanism of protection by live attenuated *Bordetella pertussis* BPZE1 against *Bordetella bronchiseptica* in mice. Vaccine 30(40):5864–5870. doi:10.1016/j.vaccine.2012.07.005

Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S, Jacobs C, van Loenhout J, de Jong D, Stunnenberg HG, Xavier RJ, van der Meer JW, van Crevel R, Netea MG (2012) Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proc Natl Acad Sci USA 109(43):17537–17542. doi:10.1073/pnas.1202870109

Kopecko DJ, Sieber H, Ures JA, Furer A, Schlup J, Knof U, Collioud A, Xu D, Colburn K, Dietrich G (2009) Genetic stability of vaccine strain *Salmonella typhi* Ty21a over 25 years. Int J Med Microbiol 299(4):233–246

Kupferschmidt K (2011) Infectious disease. Taking a new shot at a TB vaccine. Science 334 (6062):1488–1490. doi:10.1126/science.334.6062.1488

Lacombe K, Yam A, Simondon K, Pinchinat S, Simondon F (2004) Risk factors for acellular and whole-cell pertussis vaccine failure in Senegalese children. Vaccine 23(5):623–628. doi:10.1016/j.vaccine.2004.07.007

Lagos R, San Martin O, Wasserman SS, Prado V, Losonsky GA, Bustamante C, Levine MM (1999) Palatability, reactogenicity and immunogenicity of engineered live oral cholera vaccine CVD 103-HgR in Chilean infants and toddlers. Pediatr Infect Dis J 18(7):624–630

Larsen MH, Biermann K, Chen B, Hsu T, Sambandamurthy VK, Lackner AA, Aye PP, Didier P, Huang D, Shao L, Wei H, Letvin NL, Frothingham R, Haynes BF, Chen ZW, Jacobs WR Jr (2009) Efficacy and safety of live attenuated persistent and rapidly cleared *Mycobacterium tuberculosis* vaccine candidates in non-human primates. Vaccine 27(34):4709–4717. doi:10.1016/j.vaccine.2009.05.050

Lee GM, Lett S, Schauer S, LeBaron C, Murphy TV, Rusinak D, Lieu TA, Massachusetts Pertussis Study G (2004) Societal costs and morbidity of pertussis in adolescents and adults. Clin Infect Dis 39(11):1572–1580. doi:10.1086/425006

Lee WI, Huang JL, Yeh KW, Jaing TH, Lin TY, Huang YC, Chiu CH (2011) Immune defects in active mycobacterial diseases in patients with primary immunodeficiency diseases (PIDs). J Formos Med Assoc 110(12):750–758. doi:10.1016/j.jfma.2011.11.004

Levine MM, Ferreccio C, Abrego P, Martin OS, Ortiz E, Cryz S (1999) Duration of efficacy of Ty21a, attenuated *Salmonella typhi* live oral vaccine. Vaccine 17(Suppl 2):S22–S27

Levine MM, Ferreccio C, Black RE, Lagos R, San Martin O, Blackwelder WC (2007) Ty21a live oral typhoid vaccine and prevention of paratyphoid fever caused by *Salmonella enterica* Serovar Paratyphi B. Clin Infect Dis 45(Suppl 1):S24–S28

Levine MM, Kaper JB, Herrington D, Ketley J, Losonsky G, Tacket CO, Tall B, Cryz S (1988) Safety, immunogenicity, and efficacy of recombinant live oral cholera vaccines, CVD 103 and CVD 103-HgR. Lancet 2(8609):467–470

Leyten EM, Prins C, Bossink AW, Thijsen S, Ottenhoff TH, van Dissel JT, Arend SM (2007) Effect of tuberculin skin testing on a *Mycobacterium tuberculosis*-specific interferon-gamma assay. Eur Respir J 29(6):1212–1216. doi:10.1183/09031936.00117506

Liko J, Robinson SG, Cieslak PR (2013) Priming with whole-cell versus acellular pertussis vaccine. N Engl J Med 368(6):581–582

Lin FY, Vo AH, Phan VB, Nguyen TT, Bryla D, Tran CT, Ha BK, Dang DT, Robbins JB (2000) The epidemiology of typhoid fever in the Dong Thap Province, Mekong Delta region of Vietnam. Am J Trop Med Hyg 62(5):644–648

Liu D, Verma NK, Romana LK, Reeves PR (1991) Relationships among the rfb regions of *Salmonella* serovars A, B, and D. J Bacteriol 173:4814–4819

Locht C, Antoine R, Raze D, Mielcarek N, Hot D, Lemoine Y, Mascart F (2004) *Bordetella pertussis* from functional genomics to intranasal vaccination. Int J Med Microbiol 293 (7–8):583–588

Locht C, Mielcarek N (2012) New pertussis vaccination approaches: en route to protect newborns? FEMS Immunol Med Microbiol 66(2):121–133. doi:10.1111/j.1574-695X.2012.00988.x

Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY, Alvarado M, Anderson HR, Anderson LM, Andrews KG, Atkinson C, Baddour LM, Barker-Collo S, Bartels DH, Bell ML, Benjamin EJ, Bennett D, Bhalla K, Bikbov B, Bin Abdulhak A, Birbeck G, Blyth F, Bolliger I, Boufous S, Bucello C, Burch M, Burney P, Carapetis J, Chen H, Chou D, Chugh SS, Coffeng LE, Colan SD, Colquhoun S, Colson KE, Condon J, Connor MD, Cooper LT, Corriere M, Cortinovis M, de Vaccaro KC, Couser W, Cowie BC, Criqui MH, Cross M, Dabhadkar KC, Dahodwala N, De Leo D, Degenhardt L, Delossantos A, Denenberg J, Des Jarlais DC, Dharmaratne SD, Dorsey ER, Driscoll T, Duber H, Ebel B, Erwin PJ, Espindola P, Ezzati M, Feigin V, Flaxman AD, Forouzanfar MH, Fowkes FG, Franklin R, Fransen M, Freeman MK, Gabriel SE, Gakidou E, Gaspari F, Gillum RF, Gonzalez-Medina D, Halasa YA, Haring D, Harrison JE, Havmoeller R, Hay RJ, Hoen B, Hotez PJ, Hoy D, Jacobsen KH, James SL, Jasrasaria R, Jayaraman S, Johns N, Karthikeyan G, Kassebaum N, Keren A, Khoo JP, Knowlton LM, Kobusingye O, Koranteng A, Krishnamurthi R, Lipnick M, Lipshultz SE, Ohno SL, Mabweijano J, MacIntyre MF, Mallinger L, March L, Marks GB, Marks R, Matsumori A, Matzopoulos R, Mayosi BM, McAnulty JH, McDermott MM, McGrath J, Mensah GA, Merriman TR, Michaud C, Miller M, Miller TR, Mock C, Mocumbi AO, Mokdad AA, Moran A, Mulholland K, Nair MN, Naldi L, Narayan KM, Nasseri K, Norman P, O'Donnell M, Omer SB, Ortblad K, Osborne R, Ozgediz D, Pahari B, Pandian JD, Rivero AP, Padilla RP, Perez-Ruiz F, Perico N, Phillips D, Pierce K, Pope CA 3rd, Porrini E, Pourmalek F, Raju M, Ranganathan D, Rehm JT, Rein DB, Remuzzi G, Rivara FP, Roberts T, De Leon FR, Rosenfeld LC, Rushton L, Sacco RL, Salomon JA, Sampson U, Sanman E, Schwebel DC, Segui-Gomez M, Shepard DS, Singh D, Singleton J, Sliwa K, Smith E, Steer A, Taylor JA, Thomas B, Tleyjeh IM, Towbin JA, Truelsen T, Undurraga EA, Venketasubramanian N, Vijayakumar L, Vos T, Wagner GR, Wang M, Wang W, Watt K, Weinstock MA, Weintraub R, Wilkinson JD, Woolf AD, Wulf S, Yeh PH, Yip P, Zabetian A, Zheng ZJ, Lopez AD, Murray CJ (2012) Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 380(9859):2095–2128

Lugauer S, Heininger U, Cherry JD, Stehr K (2002) Long-term clinical effectiveness of an acellular pertussis component vaccine and a whole cell pertussis component vaccine. Eur J Pediatr 161(3):142–146

Mahon BP, Brady MT, Mills KH (2000) Protection against *Bordetella pertussis* in mice in the absence of detectable circulating antibody: implications for long-term immunity in children. J Infect Dis 181(6):2087–2091. doi:10.1086/315527

Marchant A, Goldman M (2005) T cell-mediated immune responses in human newborns: ready to learn? Clin Exp Immunol 141(1):10–18. doi:10.1111/j.1365-2249.2005.02799.x

Martin C, Williams A, Hernandez-Pando R, Cardona PJ, Gormley E, Bordat Y, Soto CY, Clark SO, Hatch GJ, Aguilar D, Ausina V, Gicquel B (2006) The live *Mycobacterium tuberculosis* phoP mutant strain is more attenuated than BCG and confers protective immunity against tuberculosis in mice and guinea pigs. Vaccine 24(17):3408–3419. doi:10.1016/j.vaccine.2006.03.017

Mielcarek N, Debrie AS, Mahieux S, Locht C (2010) Dose response of attenuated *Bordetella pertussis* BPZE1-induced protection in mice. Clin Vaccine Immunol 17(3):317–324. doi:10.1128/CVI.00322-09

Mielcarek N, Debrie AS, Raze D, Bertout J, Rouanet C, Younes AB, Creusy C, Engle J, Goldman WE, Locht C (2006a) Live attenuated *B. pertussis* as a single-dose nasal vaccine against whooping cough. PLoS Pathog 2(7):e65. doi:10.1371/journal.ppat.0020065

Mielcarek N, Debrie AS, Raze D, Quatannens J, Engle J, Goldman WE, Locht C (2006b) Attenuated *Bordetella pertussis*: new live vaccines for intranasal immunisation. Vaccine 24 (Suppl 2):S2-54–55

Miller D, Madge N, Diamond J, Wadsworth J, Ross E (1993) Pertussis immunisation and serious acute neurological illnesses in children. BMJ 307(6913):1171–1176

Miller DL, Ross EM, Alderslade R, Bellman MH, Rawson NS (1981) Pertussis immunisation and serious acute neurological illness in children. Br Med J (Clin Res Ed) 282(6276):1595–1599

Milstien JB, Gibson JJ (1990) Quality control of BCG vaccine by WHO: a review of factors that may influence vaccine effectiveness and safety. Bull World Health Org 68(1):93–108

Moore DL, Le Saux N, Scheifele D, Halperin SA (2004) Lack of evidence of encephalopathy related to pertussis vaccine: active surveillance by IMPACT, Canada, 1993–2002. Pediatr Infect Dis J 23(6):568–571

Mueller H, Fae KC, Magdorf K, Ganoza CA, Wahn U, Guhlich U, Feiterna-Sperling C, Kaufmann SH (2011) Granulysin-expressing CD4+ T cells as candidate immune marker for tuberculosis during childhood and adolescence. PLoS ONE 6(12):e29367. doi:10.1371/journal.pone.0029367

Nair GB, Faruque SM, Bhuiyan NA, Kamruzzaman M, Siddique AK, Sack DA (2002) New variants of *Vibrio cholerae* O1 biotype El Tor with attributes of the classical biotype from hospitalized patients with acute diarrhea in Bangladesh. J Clin Microbiol 40(9):3296–3299

Nikaido H (2003) Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67:593–656

Ohtake S, Martin R, Saxena A, Pham B, Chiueh G, Osorio M, Kopecko D, Xu D, Lechuga-Ballesteros D, Truong-Le V (2011) Room temperature stabilization of oral, live attenuated *Salmonella enterica* serovar Typhi-vectored vaccines. Vaccine 29(15):2761–2771

Olin P, Gustafsson L, Barreto L, Hessel L, Mast TC, Rie AV, Bogaerts H, Storsaeter J (2003) Declining pertussis incidence in Sweden following the introduction of acellular pertussis vaccine. Vaccine 21(17–18):2015–2021

Osorio M, Wu Y, Singh S, Merkel TJ, Bhattacharya SK, Blake MS, Kopecko OJ (2009) Anthrax protective antigen delivered by *Salmonella enterica* serovar Typhi Ty21a protects mice from a lethal anthrax spore challenge. Infect Immun 77(4):1475–1482

Ottenhoff TH, Kaufmann SH (2012) Vaccines against tuberculosis: where are we and where do we need to go? PLoS Pathog 8(5):e1002607. doi:10.1371/journal.ppat.1002607

Pakkanen SH, Kantele JM, Kantele A (2012) Cross-reactive gut-directed immune response against *Salmonella enterica* serovar Paratyphi A and B in typhoid fever and after oral Ty21a typhoid vaccination. Vaccine 30(42):6047–6053

Pasetti MF, Simon JK, Sztein MB, Levine MM (2011) Immunology of gut mucosal vaccines. Immunol Rev 239(1):125–148

Paz S, Broza M (2007) WInd direction and its linkage with *Vibrio cholerae* dissemination. Environ Health Perspect 115:195–200

Perez E, Samper S, Bordas Y, Guilhot C, Gicquel B, Martin C (2001) An essential role for phoP in *Mycobacterium tuberculosis* virulence. Mol Microbiol 41(1):179–187

Pichichero ME, Treanor J (1997) Economic impact of pertussis. Arch Pediatr Adolesc Med 151 (1):35–40

Pittman M (1991) History of the development of pertussis vaccine. Dev Biol Stand 73:13–29

Pollard R (1980) Relation between vaccination and notification rates for whooping cough in England and Wales. Lancet 1(8179):1180–1182

Poolman JT, Hallander H, Halperin SA (2011) Pertussis vaccines: where to now? Expert Rev Vaccines 10(11):1497–1500. doi:10.1586/erv.11.147

Pulickal AS, Gautam S, Clutterbuck EA, Thorson S, Basynat B, Adhikari N, Makepeace K, Rijpkema S, Borrow R, Farrar JJ, Pollard AJ (2009) Kinetics of the natural, humoral immune response to *Salmonella enterica* serovar Typhi in Kathmandu, Nepal. Clin Vaccine Immunol 16(10):1413–1419

Qualls JE, Neale G, Smith AM, Koo MS, DeFreitas AA, Zhang H, Kaplan G, Watowich SS, Murray PJ (2010) Arginine usage in mycobacteria-infected macrophages depends on autocrine-paracrine cytokine signaling. Sci Signal 3(135):ra62. doi:10.1126/scisignal.2000955

Quiambao B, Van Der Meeren O, Kolhe D, Gatchalian S (2012) A randomized, dose-ranging assessment of the immunogenicity and safety of a booster dose of a combined diphtheria-tetanus-whole cell pertussis-hepatitis B-inactivated poliovirus-Hemophilus influenzae type b (DTPw-HBV-IPV/Hib) vaccine vs. co-administration of DTPw-HBV/Hib and IPV vaccines in 12 to 24 months old Filipino toddlers. Hum Vaccin Immunother 8(3):347–354. doi:10.4161/hv.18630

Richie EE, Punjabi NH, Sidharta YY, Peetosutan KK, Sukandar MM, Wasserman SS, Lesmana MM, Wangsasaputra FF, Pandam SS, Levine MM, O'Hanley PP, Cryz SJ, Simanjuntak CH (2000) Efficacy trial of single-dose live oral cholera vaccine CVD 103-HgR in North Jakarta, Indonesia, a cholera-endemic area. Vaccine 18(22):2399–2410

Ritz N, Hanekom WA, Robins-Browne R, Britton WJ, Curtis N (2008) Influence of BCG vaccine strain on the immune response and protection against tuberculosis. FEMS Microbiol Rev 32 (5):821–841. doi:10.1111/j.1574-6976.2008.00118.x

Rivera IN, Souza KM, Souza CP, Lopes RM (2012) Free-living and plankton-associated *Vibrios*: assessment in ballast water, harbor areas, and coastal ecosystems in Brazil. Front Microbiol 3:443

Robbins G, Tripathy VM, Misra VN, Mohanty RK, Shinde VS, Gray KM, Schug MD (2009) Ancient skeletal evidence for leprosy in India (2000 B.C.). PLoS ONE 4(5):e5669. doi:10.1371/journal.pone.0005669

Roland KL, Cloninger C, Kochi SK, Thomas LJ, Tinge SA, Rouskey C, Killeen KP (2007) Construction and preclinical evaluation of recombinant Peru-15 expressing high levels of the cholera toxin B subunit as a vaccine against enterotoxigenic *Escherichia coli*. Vaccine 25 (51):8574–8584

Roth A, Gustafson P, Nhaga A, Djana Q, Poulsen A, Garly ML, Jensen H, Sodemann M, Rodriques A, Aaby P (2005) BCG vaccination scar associated with better childhood survival in Guinea-Bissau. Int J Epidemiol 34(3):540–547. doi:10.1093/ije/dyh392

Roth AE, Stensballe LG, Garly ML, Aaby P (2006) Beneficial non-targeted effects of BCG–ethical implications for the coming introduction of new TB vaccines. Tuberculosis (Edinb) 86 (6):397–403. doi:10.1016/j.tube.2006.02.001

Rumke HC, Schlumberger M, Floury B, Nagel J, van Steenis B (1993) Serological evaluation of a simplified immunization schedule using quadruple DPT-polio vaccine in Burkina Faso. Vaccine 11(11):1113–1118

Ryan M, Murphy G, Ryan E, Nilsson L, Shackley F, Gothefors L, Oymar K, Miller E, Storsaeter J, Mills KH (1998) Distinct T-cell subtypes induced with whole cell and acellular pertussis vaccines in children. Immunology 93(1):1–10

Salmaso S, Mastrantonio P, Tozzi AE, Stefanelli P, Anemona A, Ciofi degli Atti ML, Giammanco A, Stage IIIWG (2001) Sustained efficacy during the first 6 years of life of 3-component acellular pertussis vaccines administered in infancy: the Italian experience. Pediatrics 108(5):E81

Sambandamurthy VK, Derrick SC, Hsu T, Chen B, Larsen MH, Jalapathy KV, Chen M, Kim J, Porcelli SA, Chan J, Morris SL, Jacobs WR Jr (2006) *Mycobacterium tuberculosis* DeltaRD1 DeltapanCD: a safe and limited replicating mutant strain that protects immunocompetent and immunocompromised mice against experimental tuberculosis. Vaccine 24(37–39):6309–6320. doi:10.1016/j.vaccine.2006.05.097

Sambandamurthy VK, Derrick SC, Jalapathy KV, Chen B, Russell RG, Morris SL, Jacobs WR Jr (2005) Long-term protection against tuberculosis following vaccination with a severely attenuated double lysine and pantothenate auxotroph of *Mycobacterium tuberculosis*. Infect Immun 73(2):1196–1203. doi:10.1128/IAI.73.2.1196-1203.2005

Sanchez J, Holmgren J (1989) Recombinant system for overexpression of cholera toxin B subunit in *Vibrio cholerae* as a basis for vaccine development. Proc Natl Acad Sci USA 86(2):481–485

Sato H, Sato Y (1999a) Experience with diphtheria toxoid-tetanus toxoid-acellular pertussis vaccine in Japan. Clin Infect Dis 28(Suppl 2):S124–S130. doi:10.1086/515063

Sato Y, Sato H (1999b) Development of acellular pertussis vaccines. Biologicals 27(2):61–69. doi:10.1006/biol.1999.0181

Sawyer LA, McInnis J, Patel A, Horne AD, Albrecht P (1994) Deleterious effect of thimerosal on the potency of inactivated poliovirus vaccine. Vaccine 12(9):851–856

Schluger NW (2001) Recent advances in our understanding of human host responses to tuberculosis. Respir Res 2(3):157–163

Sester M, Sotgiu G, Lange C, Giehl C, Girardi E, Migliori GB, Bossink A, Dheda K, Diel R, Dominguez J, Lipman M, Nemeth J, Ravn P, Winkler S, Huitric E, Sandgren A, Manissero D (2011) Interferon-gamma release assays for the diagnosis of active tuberculosis: a systematic review and meta-analysis. Eur Respir J 37(1):100–111. doi:10.1183/09031936.00114810

Sheridan SL, Ware RS, Grimwood K, Lambert SB (2012) Number and order of whole cell pertussis vaccines in infancy and disease protection. JAMA 308(5):454–456. doi:10.1001/jama.2012.6364

Silva Miranda M, Breiman A, Allain S, Deknuydt F, Altare F (2012) The tuberculous granuloma: an unsuccessful host defence mechanism providing a safety shelter for the bacteria? Clin Dev Immunol 2012:139127. doi:10.1155/2012/139127

Simanjuntak CH, O'Hanley P, Punjabi NH, Noriega F, Pazzaglia G, Dykstra P, Kay B, Suharyono, Budiarso A, Rifai AR, et al. (1993) Safety, immunogenicity, and transmissibility of single-dose live oral cholera vaccine strain CVD 103-HgR in 24- to 59-month-old Indonesian children. J Infect Dis 168(5):1169–1176

Simon R, Tennant SM, Galen JE, Levine MM (2011) Mouse models to assess the efficacy of non-typhoidal *Salmonella* vaccines: revisiting the role of host innate susceptibility and routes of challenge. Vaccine 29(32):5094–5106

Skerry CM, Cassidy JP, English K, Feunou-Feunou P, Locht C, Mahon BP (2009) A live attenuated *Bordetella pertussis* candidate vaccine does not cause disseminating infection in gamma interferon receptor knockout mice. Clin Vaccine Immunol 16(9):1344–1351. doi:10.1128/CVI.00082-09

Skowronski DM, De Serres G, MacDonald D, Wu W, Shaw C, Macnabb J, Champagne S, Patrick DM, Halperin SA (2002) The changing age and seasonal profile of pertussis in Canada. J Infect Dis 185(10):1448–1453. doi:10.1086/340280

Steinhoff MC, Reed GF, Decker MD, Edwards KM, Englund JA, Pichichero ME, Rennels MB, Anderson EL, Deloria MA, Meade BD (1995) A randomized comparison of reactogenicity and immunogenicity of two whole-cell pertussis vaccines. Pediatrics 96(3 Pt 2):567–570

Stojanov S, Liese J, Belohradsky BH (2000) Hospitalization and complications in children under 2 years of age with *Bordetella pertussis* infection. Infection 28(2):106–110

Storsaeter J, Wolter J (2006) Is there a need for a new generation of vaccines against pertussis? Expert Opin Emerg Drugs 11(2):195–205. doi:10.1517/14728214.11.2.195

Storsaeter J, Wolter J, Locht C (2007) Pertussis vaccines. In: Locht C (ed) Bordetella molecular microbiology. Horizon Bioscience, UK

Sun R, Skeiky YA, Izzo A, Dheenadhayalan V, Imam Z, Penn E, Stagliano K, Haddock S, Mueller S, Fulkerson J, Scanga C, Grover A, Derrick SC, Morris S, Hone DM, Horwitz MA, Kaufmann SH, Sadoff JC (2009) Novel recombinant BCG expressing perfringolysin O and the over-expression of key immunodominant antigens; pre-clinical characterization, safety and protection against challenge with *Mycobacterium tuberculosis*. Vaccine 27(33):4412–4423. doi:10.1016/j.vaccine.2009.05.048

Sur D, Kanungo S, Sah B, Manna B, Ali M, Paisley AM, Niyogi SK, Park JK, Sarkar B, Puri MK, Kim DR, Deen JL, Holmgren J, Carbis R, Rao R, Nguyen TV, Han SH, Attridge S, Donner A, Ganguly NK, Bhattacharya SK, Nair GB, Clemens JD, Lopez AL (2011) Efficacy of a low-cost, inactivated whole-cell oral cholera vaccine: results from 3 years of follow-up of a randomized, controlled trial. PLoS Negl Trop Dis 5(10):e1289

Sutherland I, Lindgren I (1979) The protective effect of BCG vaccination as indicated by autopsy studies. Tubercle 60(4):225–231

Svennerholm AM (2011) From cholera to enterotoxigenic *Escherichia coli* (ETEC) vaccine development. Indian J Med Res 133:188–196

Svennerholm AM, Holmgren J (1976) Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin/toxoid. Infect Immun 13(3):735–740

Sweeney KA, Dao DN, Goldberg MF, Hsu T, Venkataswamy MM, Henao-Tamayo M, Ordway D, Sellers RS, Jain P, Chen B, Chen M, Kim J, Lukose R, Chan J, Orme IM, Porcelli SA, Jacobs WR Jr (2011) A recombinant *Mycobacterium smegmatis* induces potent bactericidal immunity against *Mycobacterium tuberculosis*. Nat Med 17(10):1261–1268. doi:10.1038/nm.2420

Tacket CO, Cohen MB, Wasserman SS, Losonsky G, Livio S, Kotloff K, Edelman R, Kaper JB, Cryz SJ, Giannella RA, Schiff G, Levine MM (1999) Randomized, double-blind, placebo-controlled, multicentered trial of the efficacy of a single dose of live oral cholera vaccine CVD 103-HgR in preventing cholera following challenge with *Vibrio cholerae* O1 El tor inaba three months after vaccination. Infect Immun 67(12):6341–6345

Tacket CO, Levine MM (2007) CVD 908, CVD 908-htrA, and CVD 909 live oral typhoid vaccines: a logical progression. Clin Infect Dis 45(Suppl 1):S20–S23

Tennant SM, Wang JY, Galen JE, Simon R, Pasetti MF, Gat O, Levine MM (2011) Engineering and preclinical evaluation of attenuated nontyphoidal *Salmonella* strains serving as live oral vaccines and as reagent strains. Infect Immun 79(10):4175–4185

Therre H, Baron S (2000) Pertussis immunisation in Europe—the situation in late 1999. Euro Surveill 5(1):6–10

Tomovasu T, Ohkishi T, Ukyo Y, Tokumitsu A, Takaya A, Suzuki M, Sekiya K, Matsui H, Kutsukake K, Yomamoto T (2002) The ClpX ATP-dependent protease regulates flagellum synthesis in *Salmonella enterica* serovar Typhimurium. J Bacteriol 184(3):645–653

Tran TH, Nguyen TD, Nguyen TT, Ninh TT, Tran NB, Nguyen VM, Tran TT, Cao TT, Pham VM, Nguyen TC, Tran TD, Pham VT, To SD, Campbell JI, Stockwell E, Schultsz C, Simmons CP, Glover C, Lam W, Marques F, May JP, Upton A, Budhram R, Dougan G, Farrar J, Nguyen VV, Dolecek C (2010) A randomised trial evaluating the safety and immunogenicity of the novel single oral dose typhoid vaccine M01ZH09 in healthy Vietnamese children. PLoS ONE 5(7):e11778

Trunz BB, Fine P, Dye C (2006) Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. Lancet 367(9517):1173–1180. doi:10.1016/S0140-6736(06)68507-3

Vekemans J, Amedei A, Ota MO, D'Elios MM, Goetghebuer T, Ismaili J, Newport MJ, Del Prete G, Goldman M, McAdam KP, Marchant A (2001) Neonatal bacillus Calmette-Guerin vaccination induces adult-like IFN-gamma production by CD4+ T lymphocytes. Eur J Immunol 31(5):1531–1535. doi:10.1002/1521-4141(200105)31:5<1531:AID-IMMU1531>3.0.CO;2-1

Venkataswamy MM, Goldberg MF, Baena A, Chan J, Jacobs WR Jr, Porcelli SA (2012) In vitro culture medium influences the vaccine efficacy of *Mycobacterium bovis* BCG. Vaccine 30(6):1038–1049. doi:10.1016/j.vaccine.2011.12.044

Verreck FA, Vervenne RA, Kondova I, van Kralingen KW, Remarque EJ, Braskamp G, van der Werff NM, Kersbergen A, Ottenhoff TH, Heidt PJ, Gilbert SC, Gicquel B, Hill AV, Martin C, McShane H, Thomas AW (2009) MVA.85A boosting of BCG and an attenuated, phoP deficient *M. tuberculosis* vaccine both show protective efficacy against tuberculosis in rhesus macaques. PLoS ONE 4(4):e5264. doi:10.1371/journal.pone.0005264

Vogel FR, Leclerc C, Schutze MP, Jolivet M, Audibert F, Klein TW, Chedid L (1987) Modulation of carrier-induced epitopic suppression by *Bordetella pertussis* components and muramyl peptide. Cell Immunol 107(1):40–51

von Konig CH, Halperin S, Riffelmann M, Guiso N (2002) Pertussis of adults and infants. Lancet Infect Dis 2(12):744–750

von Reyn CF, Mtei L, Arbeit RD, Waddell R, Cole B, Mackenzie T, Matee M, Bakari M, Tvaroha S, Adams LV, Horsburgh CR, Pallangyo K, DarDar Study G (2010) Prevention of tuberculosis in bacille Calmette-Guerin-primed, HIV-infected adults boosted with an inactivated whole-cell mycobacterial vaccine. AIDS 24(5):675–685. doi:10.1097/QAD.0b013e3283350f1b

Wahid R, Pasetti MF, Maciel M Jr, Simon JK, Tacket CO, Levine MM, Sztein MB (2011) Oral priming with *Salmonella typhi* vaccine strain CVD 909 followed by parenteral boost with the *S. typhi* Vi capsular polysaccharide vaccine induces CD27+ IgD-*S. typhi*-specific IgA and IgG B memory cells in humans. Clin Immunol 138(2):187–200

Wahid R, Simon R, Zafar SJ, Levine MM, Sztein MB (2012) Live oral typhoid vaccine Ty21a induces cross-reactive humoral immune responses against *Salmonella enterica* serovar Paratyphi A and *S. paratyphi* B in humans. Clin Vaccine Immunol 19(6):825–834

Walker AM, Jick H, Perera DR, Knauss TA, Thompson RS (1988) Neurologic events following diphtheria-tetanus-pertussis immunization. Pediatrics 81(3):345–349

Walker V, Selby G, Wacogne I (2006) Does neonatal BCG vaccination protect against tuberculous meningitis? Arch Dis Child 91(9):789–791. doi:10.1136/adc.2006.098459

Wang JY, Pasetti MF, Noriega FR, Anderson RJ, S.S. W, Galen JE, Sztein MB, Levine MM (2001) Construction, genotypic and phenotypic characterization, and immunogenciity of attenated DeltaguaBA *Salmonella enterica* serover Typhi strain CVD 915. Infect Immun 69 (8):4734–4741

Watkins ML, Semple PL, Abel B, Hanekom WA, Kaplan G, Ress SR (2008) Exposure of cord blood to *Mycobacterium bovis* BCG induces an innate response but not a T-cell cytokine response. Clin Vaccine Immunol 15(11):1666–1673. doi:10.1128/CVI.00202-08

Weingart CL, Keitel WA, Edwards KM, Weiss AA (2000) Characterization of bactericidal immune responses following vaccination with acellular pertussis vaccines in adults. Infect Immun 68(12):7175–7179

Wendelboe AM, Van Rie A, Salmaso S, Englund JA (2005) Duration of immunity against pertussis after natural infection or vaccination. Pediatr Infect Dis J 24(5 Suppl):S58–S61

Winter K, Harriman K, Zipprich J, Schechter R, Talarico J, Watt J, Chavez G (2012) California pertussis epidemic, 2010. J Pediatr. doi:10.1016/j.jpeds.2012.05.041

Witt MA, Katz PH, Witt DJ (2012) Unexpectedly limited durability of immunity following acellular pertussis vaccination in preadolescents in a North American outbreak. Clin Infect Dis 54(12):1730–1735. doi:10.1093/cid/cis287

Woodworth JS, Wu Y, Behar SM (2008) *Mycobacterium tuberculosis*-specific CD8+ T cells require perforin to kill target cells and provide protection in vivo. J Immunol 181 (12):8595–8603

World Health Organization (2001) Cholera vaccines. WHO position paper. WHO Wkly Epidemiol Rec 76(16):117–124

World Health Organization (2004) BCG vaccine. WHO position paper. Wkly Epidemiol Rec 79 (4):27–38

World Health Organization (2005) Pertussis vaccines–WHO position paper. Wkly Epidemiol Rec 80(4):31–39

World Health Organization (2010) Global Advisory Committee on vaccine safety, 3–4 December 2009. Wkly Epidemiol Rec 85(5):29–33

World Health Organization (2011) TB/HIV Facts 2011–2012. http://www.who.int/tb/publications/TBHIV_Facts_for_2011.pdf

World Health Organization (2012a) Global tuberculosis report, 2012

World Health Organization (2012b) Immunization summary: a statistical reference containing data through 2010

World Health Organization (2012c) WHO prequalified vaccines. In: Immunization Standards. World Health Organization. http://www.who.int/immunization_standards/vaccine_quality/PQ_vaccine_list_en/en/index.html

World Health Organization (2012d) WHO Technical Working Group on creation of an oral cholera vaccine stockpile. In: Diseases PaE (ed) Meeting report, 26–27 April 2012. WHO, Geneva

Xu DQ, Zhang L, Kopecko DJ, Gao L, Shao Y, Guo B et al (2009) Bacterial delivery of siRNAs: a new approach to solid tumor therapy. Methods Mol Biol 487:161–187

Yang XY, Chen QF, Li YP, Wu SM (2011) *Mycobacterium vaccae* as adjuvant therapy to anti-tuberculosis chemotherapy in never-treated tuberculosis patients: a meta-analysis. PLoS ONE 6 (9):e23826. doi:10.1371/journal.pone.0023826

Zepp F, Heininger U, Mertsola J, Bernatowska E, Guiso N, Roord J, Tozzi AE, Van Damme P (2011) Rationale for pertussis booster vaccination throughout life in Europe. Lancet Infect Dis 11(7):557–570. doi:10.1016/S1473-3099(11)70007-X

Zhang L, Prietsch SO, Axelsson I, Halperin SA (2011) Acellular vaccines for preventing whooping cough in children. Cochrane Database Syst Rev 1:CD001478

Zhang L, Prietsch SO, Axelsson I, Halperin SA (2012) Acellular vaccines for preventing whooping cough in children. Cochrane Database Syst Rev 3:CD001478. doi:10.1002/14651858.CD001478.pub5

Zhou L, Pollard AJ (2010) A fast and highly sensitive blood culture PCR method for clinical detection of *Salmonella enterica* serovar Typhi. Ann Clin Microbiol Antimicrob 9:14

Zorlu G (2012) Cholera vaccine deployed to control African outbreak. Nature 486(7402):157–286

Chapter 6
Analytical Control Strategy of Bacterial Subunit Vaccines

Shwu-Maan Lee, Vincent Turula, Bradford Powell, Mikkel Nissum, Mariagrazia Pizza, Claudia Magagnoli and Niranjan M. Kumar

6.1 Introduction

The success of vaccines and their positive impact on public health cannot be overstated. Vaccines have evolved through history and can be classified into two broad groups by composition, which generally relates to their chronology of discovery. Traditional vaccines are based on the administration of either live, attenuated organisms, or inactivated/killed organisms. Vaccines in this group include well-recognized products such as cowpox (for preventing smallpox), varicella (chickenpox), MMR (measles, mumps, rubella), IPV (polio), hepatitis A, and the seasonal flu (influenza) vaccines. These attenuated or killed whole microbe type of preparations helped to establish the early notion of immune prophylaxis, which gave rise to modern vaccinology beginning in the nineteenth century with the famous early studies of microbial pathogens, infectious diseases, and new medical treatments. New treatments appearing in this period included a second type of

Present Address:
S.-M. Lee
PATH Malaria Vaccine Initiative, 455 Massachusetts Avenue, NW Suite 1000, Washington, DC 20001-2621, USA

V. Turula
Pfizer Inc., 401 N, Middletown Road, Pearl River, New York, NY, USA

B. Powell
PharmAthene Inc., One Park Place, Suite 450, Annapolis, MD, USA

M. Nissum · M. Pizza · C. Magagnoli
Novartis Vaccines, Via Fiorentina 1, 53100 Siena, Italy

N.M. Kumar (✉)
American Biosources Inc., 80 Main Street, 5th Floor, West Orange, NJ, USA
e-mail: nkumar.wharton@gmail.com

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6_6

vaccine developed against epidemic bacterial microbes including cholera (caused by *Vibrio cholerae*), chicken cholera (*Pasteurella munticoda*), and anthrax (*Bacillus anthracis*). This second group of vaccines is based on the administration of inactive microbial components, which were originally classified as toxoid-type vaccines as they principally contained inactivated bacterial toxins. Their development coincides with the discoveries of serum-derived treatments (antitoxin; i.e., immune therapy) against the devastating childhood diseases of diphtheria and bacterial influenza. Today, the general heading of subunit vaccines includes products comprising purified protein antigen, sometimes covalently bound to microbe-specific carbohydrate, and nearly always including an adjuvant of some type to enhance immune activity. Products for immune therapy such as monoclonal antibodies have grown into the largest class of biological products, and are not generally used as prophylactic vaccines (however, see Chap. 17 for one exception which targets immune regulators called dendritic cells). This chapter concerns a few of the most prominent subunit and toxoid type vaccine products.

There are several types of subunit bacterial vaccines, and all employ extracted, purified, or engineered components from one or more pathogens. These are purified substances that range from secreted proteins, membrane proteins, outer vesicles membranes, capsular polysaccharides, or inactive toxin or toxin fragments (Levine 2004; Plotkin et al. 2008; Hilleman 2000). For toxoid-type vaccines, the immunogenic components are chemically or genetically modified so as to remove toxic activity (Galazka 1993). The recombinant subunit approach has become popular for toxoid and nontoxin antigens, wherein a gene of the target vaccine is transferred to a non-pathogenic expression organism, expressed, purified, and tested (Liljeqvist and Ståhl 1999a, b; Gentschev et al. 2001). Pertussis toxoid, produced by *Bordetella pertussis* with specific mutations in its toxin gene, is included as an active component of an acellular Pertussis (aP) vaccine (Del Giudice et al. 1998; Del Giudice and Rappuoli 1999). A recent and effective type of bacterial derived subunit vaccine is the surface protein from the outer membrane vesicles (OMV) of Gram-negative bacteria (Rinaudo et al. 2009; Rappuoli 2007). Production occurs via fermentation, either a recombinant expressed or purified subunit protein vaccine in a heterologous expression system (e.g., bacteria or yeast) or purified from large amounts of the pathogenic organism (Plotkin et al. 2008). In general, with bacterial derived protein vaccines both universal and strain specific, such as in Meningitides serogroup B vaccine design have been pursued (Rinaudo et al. 2009). Compared to traditional whole-cell preparations, subunit vaccines contain fewer antigens and simpler total composition. This well-defined and pure composition is intended to improve the safety profile, analytical testing, and regulatory review of a subunit vaccine as a new drug entity (Levine 2004). However, the loss of multiple antigens often compromises immunogenicity in scope and longevity. Consequently, subunit products require additional immune stimulation in the form of an adjuvant, such as aluminum salts (Newman and Powell 1995).

In this chapter, the analytical control strategy supporting bacterial subunit derived proteins is described using a few licensed and new products as examples. General descriptions and details of the critical assays are presented for the well-established licensed vaccines of Diphtheria, Tetanus, acellular Pertussis toxoid

vaccines (DTaP), and DTaP combination vaccine, plus more recent Cholera and Meningococcal B OMV products, as well as the existing and next generation subunit Anthrax vaccines.

6.2 Diphtheria, Tetanus, Acellular Pertussis Toxoid Vaccines, and DTaP Combination Vaccine

This section summarizes testing principles and strategy for diphtheria, tetanus, aP toxoid vaccines, and DTaP combination vaccine. DTP is the first combination vaccine used to immunize infants and children in 1948. Over the years, other vaccines were added to the combination and whole-cell pertussis antigen was replaced with less reactogenic aP antigens (Skibinski et al. 2011).

The testing of toxoid vaccines presents a challenge; chemical inactivation creates heterogeneity in the product and it is difficult to characterize biochemically, even using state-of-the-art techniques. Thus, the analytical strategy supporting the qualification of toxoid vaccines is generally composed of classical methods given their reliability of performance.

Analysis of diphtheria and tetanus vaccines includes testing of toxin and toxoid. Toxin tests include measurement of antigen content by limit of flocculation (Lf), toxicity of the purified toxin before inactivation (integrity), total protein by protein nitrogen, and purity based on antigen content and protein nitrogen. Toxoid tests include residual formaldehyde inactivation agent, residual process impurities (ammonium and sulfate), potency, specific toxicity to verify the completeness of inactivation and irreversibility to assure toxoid does not revert back to be toxic upon storage. Most of the methods are available in the European Pharmacopeia (EP), WHO technical reports and NIH guidance documents. In some instances, a comparison of these three references is provided. The aP vaccine described here is a single-component pertussis toxoid. Testing of pertussis toxin (PT) with respect to purity and biological activity includes SDS-PAGE, HPLC, Western blot, process-related impurities (blue dye and fetuin), hemagglutination, ADP-ribosylation, and Chinese Hamster Ovary (CHO) toxicity. Testing of unadsorbed pertussis toxoid includes measurement of residual hemagglutination, inactivation kinetics, CHO toxicity, and residual inactivation agent, peroxide. Testing of adsorbed toxoid includes histamine sensitization assay (HIST), reversion to toxicity, potency, and percent adsorption. Testing of DTaP combination includes percent adsorption, identity, specific toxicity, general safety, formaldehyde, aluminum, endotoxin, and thimerosal. Thimerosal as a preservative for human formulations was removed or reduced to trace amounts in all vaccines for children 6 years of age and younger (CDC 2012). However, the preponderance of available evidence has failed to demonstrate serious harm associated with thimerosal in vaccines (Orenstein et al. 2013).

The ongoing effort to establish international reference standards and to standardize assays for vaccine evaluation is also summarized.

6.2.1 Tetanus Toxoid Vaccine

Tetanus vaccine is used to prevent a deadly tetanus disease characterized by convulsive tonic contractions of voluntary muscles. The causative agent is a Gram-positive, spore-forming, strict anaerobic microorganism, *Clostridium tetani*. Tetanus toxin is an exotoxin with a heavy chain of about 100 kDa and a light chain of about 50 kDa linked by disulfide bonds. The most widely used chemical detoxification reagent is formaldehyde; the treatment results in intramolecular and intermolecular cross-linking (Wirz et al. 1990).

The production of tetanus toxoid vaccine involves the following steps:

1. Production of tetanus toxin by fermentation of *C. tetani.*
2. Purification of tetanus toxin; ammonium sulfate fractionation is the most common method.
3. Inactivation of toxin by formaldehyde treatment.

A list of testing for tetanus vaccine is outlined in Table 6.1.

Table 6.1 Testing for tetanus vaccine intermediate (toxin), bulk, final bulk, and final containers

Material	General tests	Product specific tests
Toxin	Bioburden	Antigen concentration by limit of flocculation (Lf/mL)[a]
		Toxicity of purified toxin (L+/10 or L+)
		Minimum lethal dose (MLD)
		Antigen purity (Lf/mg protein nitrogen)
Bulk purified toxoid	pH	Antigen concentration by Lf/mL[a]
	Endotoxin	Characterization (SDS-PAGE)
	Sterility	Antigen purity (Lf/mg protein nitrogen)
	Appearance	Process-related impurities (e.g., ammonium and sulfate)
		Residual inactivation agent (formaldehyde)
		Absence of toxin and irreversibility of toxoid
		Potency (tested as adsorbed toxoid)[a]
Adsorbed toxoid (final bulk and containers)	Aluminum	Potency[a]
	pH	Identity[a]
	Sterility	% Adsorption[a]
	Thimerosal (if applicable)	Specific toxicity
	General safety	

[a] The step-by-step procedure is included in a WHO publication (WHO 2013b)

6.2.1.1 Antigen Content by Limit of Flocculation (Lf), Ramon Assay (Ph. Eur. 2.7.27)

The content of toxin or toxoid in a sample can be expressed as a flocculation value using the Ramon assay. In this assay, antitoxin is added in increasing concentrations to series of tubes containing a constant amount of toxin or toxoid. At the equivalence point of toxin/toxoid and antitoxin, flocculation occurs in one or more tubes. The first tube in which flocculation occurs is used to determine the Lf value of the sample.

Lf refers to the amount of toxin or toxoid that when reacted with 1 international unit of antitoxin gives a flocculation in the shortest period of time. The Lf value shall be determined by comparison with a reference material calibrated against the International Reference Reagent of Tetanus Toxoid for flocculation test or approved equivalent (WHO 1990).

6.2.1.2 Toxicity of Purified Tetanus Toxin (L+/10, L+ and MLD)

L+/10 is defined as the minimum amount of toxin which, when mixed with 0.1 IU of antitoxin kills an animal of a defined weight in 4 days. L+ is defined as the minimum amount of toxin which when combined with 1 IU of antitoxin kills an animal of a defined weight in 4 days (WHO 1965). MLD is the smallest amount of toxin that, when injected by a given route, causes death of the treated animals within a stated period of time (Wirz et al. 1990).

6.2.1.3 Antigen Purity (Lf/Mg Protein Nitrogen)

Purity (or specific activity) is expressed as Lf/mg protein nitrogen and WHO requirement is a minimum value of 1,000 (WHO 2014a).

Protein nitrogen is determined by the micro-Kjeldahl method. The proteins are first precipitated with 5 % trichloroacetic acid (WHO 1965) and subsequently, the precipitate is collected and washed. The Kjeldahl method starts with a sulfuric acid digestion to convert nitrogen in the sample to ammonium hydrogen sulfate. Upon alkaline addition, the liberating ammonium is distilled into HCl and titrated with NaOH (Ph. Eur. 2.5.9). Without precipitation, the micro-Kjeldahl determines the total nitrogen.

6.2.1.4 Characterization by SDS-PAGE

SDS-PAGE for tetanus toxoid can only be used qualitatively for purity assessment due to cross-linking to itself and to the impurities during formaldehyde inactivation. Figure 6.1 shows tetanus toxoid under reducing conditions. The dominant band on the gel is a tetanus toxoid monomer which migrated between 200 and 116 kDa

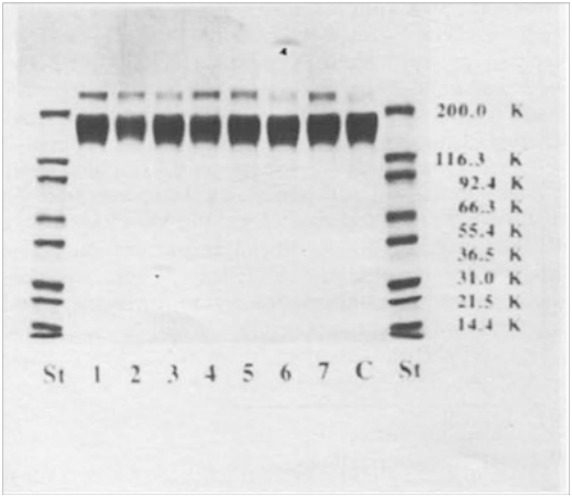


Fig. 6.1 SDS-PAGE analysis of tetanus toxoid samples under reducing conditions. Tetanus toxoid before (*lane 1*) and after treatment (*lanes 2–8*) under various experimental conditions for microencapsulation. Reproduced with permission from Elsevier (Alonso et al. 1994)

molecular weight standards. The reducing conditions did not separate the heavy and light chains because they have been cross-linked during formaldehyde treatment. The high molecular weight cross-linked molecules are also visible in most of the preparations.

6.2.1.5 Process-Related Impurity: Ammonium and Sulfate

Ammonium forms colored complex with basic tetraiodine mercurate. A limit assay is established by comparing color intensity of test sample with 1 ppm ammonium standard after complex formation (Ph. Eur. 2.4.1 Method A).

Sulfate is precipitated with barium chloride in acid. After precipitation, the opalescence of test sample and 10 ppm of sulfate standard (potassium sulfate) is compared (Ph. Eur. 2.4.13).

6.2.1.6 Absence of Tetanus Toxin and Irreversibility of Toxoid

The bulk-purified TT should be tested to ensure that reversion to toxicity cannot take place on storage. The purified bulk toxoid should be diluted in order to obtain the same concentration as that present in the final bulk except the presence of adjuvant. Divide the dilution in two equal parts. Keep one part at 2–8 °C and the other at 37 °C for 6 weeks. Both dilutions are tested in the same way. A total of 15 guinea pigs, each weighing 250–350 gm and that have not previously been treated with any material that will interfere with the test, is used in the test. Five milli Litre of each dilution is injected subcutaneously into two groups of five guinea pigs. The additional five guinea pigs should receive subcutaneously at least 500 Lf of the

Table 6.2 Comparison of three guidelines for the absence of tetanus toxin and irreversibility of toxoid

	Ph. Eur. 0452	WHO (2014a)	US (NIH 1952)
Irreversibility of toxoid	Toxoid tested at the same concentration as final bulk (e.g., 12 Lf/mL) and kept at 37 and 5 °C for 6 weeks. Each of 5 guinea pigs receives 5 mL of toxoid. Observe for 3 weeks	Section A 3.4.5: For bulk purified toxoid, similar to EP	Not described
Absence of toxin	Guinea pigs are injected with 1 mL of 500 Lf of toxoid and observed for 21 days for sign of tetanus intoxication	Section A 3.4.4: Bulk purified toxoid follows the same procedure as Ph. Eur.	Section 2.2: Inject subcutaneously into at least 4 guinea pigs weighing 300–400 gram ≥5 single human doses (≥2.0 mL) produced no symptoms of tetanus toxin poisoning for 21 days

nonincubated bulk purified toxoid in a volume of 1 mL (to test for the absence of toxin). The bulk-purified toxoid complies with the test if during the 21 days following the injection no animal shows signs of or dies from tetanus. If more than one animal dies from nonspecific causes, the test may be repeated; if more than one animal dies in the second test, the toxoid does not comply with the test (Ph. Eur. 0452). The procedure varies slightly in different guidelines; however, they follow the same general principles (WHO 2014a; NIH 1952). A comparison is provided in Table 6.2.

6.2.1.7 Specific Toxicity

This method is included in Ph. Eur. 0452 under production, general provisions, specific toxicity for adsorbed tetanus toxoid vaccine. It is also cited in Section A 3.5.2.5 of WHO document (2014a) for final bulk. Inject subcutaneously at least five single human doses (SHDs) to each of the five guinea pigs and observe 21 days for paralysis. The specific toxicity test for final bulk may be omitted once consistency of manufacturing is established (WHO 2014a).

6.2.1.8 Potency for Adsorbed Tetanus Toxoid

Potency is determined by administration of the vaccine to animals (guinea pigs or mice) followed either by challenge with tetanus toxin (method A or B) or by determination of antitoxin in the serum of the guinea pigs (method C). Methods

A or B are used during development and whenever revalidation is needed following significant manufacturing process changes. Method C is used in the interest of animal welfare. The potency of the vaccine should be determined by comparison with an appropriate reference material calibrated against the International Standard for tetanus toxoid adsorbed (Ph. Eur. 2.7.8).

The WHO International Standard preparation of tetanus toxoid adsorbed has been defined in IU based on the results obtained in guinea pig challenge assays. The 4th International Standard for Tetanus Toxoid adsorbed was established in October 2010 to have a unitage of 490 IU/ampoule (Tierney et al. 2011).

Method A: Challenge test in guinea pigs

For each assay, three groups of animals (sufficient number of animals to obtain a valid assay) are immunized subcutaneously with three twofold to 2.5-fold dilutions of the vaccine under test and three groups are given three analogous dilutions of the standard. The dilutions were made in saline. After 28 days, animals are given a paralytic or lethal challenge with 1 mL of toxin containing 50 LD_{50} per animal. Dilutions of both preparations must be such that the intermediate dose protects about 50 % of the animals. The number of guinea pigs without paralysis should be counted 5 days after injection of the challenge toxin. Results of the assay are then statistically analyzed for determination of vaccine potency.

Method B: Challenge test in mice.

Method B is very similar to method A, except 5-week-old mice are used, the immunization dose is 0.5 mL and the challenge dose is 50 LD_{50} in 0.5 mL, given after 28 days. The number of mice without paralysis is counted 4 days after challenge.

Method C: Determination of antibodies in guinea pigs

One mL of the vaccine or reference standard dilutions are injected subcutaneously to each guinea pig, a blood sample is obtained 35–42 days later, and the relative antibody titer is determined by (1) Enzyme-linked immunosorbent assay (ELISA) or (2) toxin-binding inhibition assay.

Method C (1) ELISA plates are coated with tetanus toxoid and peroxidase–conjugated rabbit or goat antibody against guinea pig IgG is used in detection.

Method C (2) For toxin–binding inhibition assay, tetanus toxin or toxoid is added to serial dilutions of test and reference sera; the serum/antigen mixtures are incubated overnight. The unbound toxin or toxoid is determined by ELISA with plates coated with antitoxin and peroxidase-conjugated equine anti-tetanus IgG is used for detection.

All the three above methods are multi-dilution assays, which should be used to establish production consistency, product shelf life, and calibrate reference preparation. Potency for lot release following licensing can be simplified as a one-dilution assay. Multiple-dilution assay is carried out only periodically, or when manufacturing changes occur with the need to justify to the regulatory authorities.

The single dilution assay involves the selection of a dose of the reference vaccine, expressed as a fraction of 40 IU (SHD), that elicits a minimal protective effect and comparing its effect with the response elicited by the same fraction of a human dose of the test vaccine. If the response to the test vaccine is significantly greater than the response to the reference vaccine, the potency of the test vaccine is satisfactory (WHO 2014a).

The approach taken by the USA is described in the "Minimum Requirements," often referred to as the NIH method (NIH 1952). Minimal acceptable potency in these documents is defined as the capacity of a test dose of vaccine to induce an antibody response that reaches or surpasses the threshold of 2 units/mL. A suitable reference antitoxin, to which "units/mL" have been assigned, is used to control the activity of the test toxin, which is used to measure the neutralizing activity of the sera induced in guinea pigs, in an in vivo assay. The US potency assay development history was reviewed, together with an overview of the possibility of using alternative, nonanimal methods to measure neutralization in the assay. Toxin neutralization in vivo is correlated with biological protection, but antigen antibody binding by ELISA in vitro could not distinguish between neutralizing antibody and nonneutralizing antibody (Keller 2011). The EP, WHO, and US methods are briefly summarized in Table 6.3. For routine lot release, validated serological assays are being used that offer significant advantages in terms of reduction in animal numbers (Stickings et al. 2011). The test methods are generally proposed by the manufacturer and evaluated by the regulatory authorities; there are no universally accepted methods.

Table 6.3 Summary of potency assay for adsorbed tetanus vaccine from different guidelines

EP (Ph. Eur. 2.7.8)	WHO (2013b, 2014a)	USA (NIH 1952)
Immunize groups of guinea pigs or mice with test and reference vaccines. After 28 days, challenge animals with tetanus toxin and observe tetanus paralysis for 5 days (guinea pigs) or 4 days (mice). Alternatively, at 35–42 days, test sera by ELISA or toxin binding inhibition assay. Compare results with reference standard calibrated in international units	Same as Ph. Eur.	Immunize four or more guinea pigs with 1 SHD of vaccine and collect the immune serum 6 weeks later. A defined dilution of immune serum pool is combined with a standardized quantity of tetanus toxin and then injected into test animals. Protection of test animals, which equates to toxoid potency, is determined using two guinea pigs or three mice. The two control guinea pigs injected with toxin must die in 4 days, whereas the test animals should survive for 96 h or more
	The potency of tetanus vaccine used for the immunization of children should be ≥40 IU/single human dose	Minimum requirement is ≥2 antitoxin units/mL

6.2.2 Diphtheria Toxoid (DT)

Diphtheria is an acute, often fatal bacterial disease caused by toxigenic strains of *Corynebacterium diphtheriae*. Diphtheria toxin is the major virulence factor of these microorganisms and the clinical manifestations of the disease are due mainly to the presence of circulating toxin in the bloodstream of the infected individuals. Active immunization against diphtheria is based on the use of formaldehyde-detoxified preparation of diphtheria toxoid (DT). The production of Diphtheria vaccine utilizes similar methods as those described in Sect. 6.2.1.

Diphtheria toxin is secreted by *C. diphtheriae* as a single polypeptide proenzyme of 58,342 Da, but purified preparations of toxin frequently contain a heterogeneous array of molecular species. In most preparations, a fraction of the toxin has been cleaved by contaminating trypsin-like proteases, yielding a nicked form containing two fragments: A (21.2 kDa) and B (37.2 kDa), held together by a disulfide bridge. It is the nicked form that is believed to be responsible for the biological effects of diphtheria toxin. Fragment A is a potent ADP-ribosylating enzyme and fragment B recognizes mammalian cell surface receptors. Formaldehyde detoxification cross-links the two fragments and stabilizes the molecule (Carroll et al. 1988; Pappenheimer 1984). Diphtheria toxin has the capacity to block protein synthesis in cultured mammalian cells, thus causing cell death. This capacity was utilized for an in vitro neutralization assay to determine antibody titer using Vero cells. Table 6.4 outlines the testing strategy for diphtheria vaccine.

6.2.2.1 SDS-PAGE of Diphtheria Toxin and Toxoid

Diphtheria toxin and 10 experimental toxoids were analyzed on a 10 % reducing SDS-PAGE (Fig. 6.2). The diphtheria toxin used in this study was almost completely nicked. The bands of the A and B fragments appeared at higher apparent masses (27 and 43 kDa, respectively). Higher formaldehyde concentrations increased the intensity of the 58-kDa toxoid band by cross-linking of the A and B fragments and broadened the band. In the meantime, formaldehyde shifted the A and B fragments to lower molecular weight species (Metz et al. 2003)

6.2.2.2 Antigen Content by Limit of Flocculation (Lf)

The quantitation is based on flocculation of toxoid and antitoxin at the equivalence point. The test is carried out with antiserum calibrated against the WHO reference reagent of DT for flocculation test. The Lf dose of toxin is equivalent to about 2.5 μg protein (Pappenheimer 1984).

Table 6.4 Summary of testing for diphtheria vaccine including toxin intermediate and final product

	General test	Product specific test
Toxin	Bioburden	Antigen concentration by Lf (Lf/mL)[a]
		Toxicity (L+/10 or L+)
		Minimal lethal dose (MLD)
		Antigen purity (Lf/mg protein nitrogen)
		SDS-PAGE
Bulk purified toxoid	pH	SDS-PAGE
	Endotoxin	Antigen concentration by Lf (Lf/mL)[a]
	Sterility	Antigen purity (Lf/mg protein nitrogen)
	Appearance	Process-related impurities (ammonium and sulfate)
		Inactivation agent (formaldehyde)
		Specific toxicity
		Absence of toxin and irreversibility of toxoid[a]
		Potency[a] (tested adsorbed)
Adsorbed toxoid (final bulk and containers)	Aluminum	Potency[a] (if not already performed)
	Thimerosal (if applicable)	Identity[a]
	pH	% Adsorption[a]
	Sterility	Specific toxicity (if not performed earlier)

[a] The step-by-step procedure is included in a WHO publication (WHO 2013b)

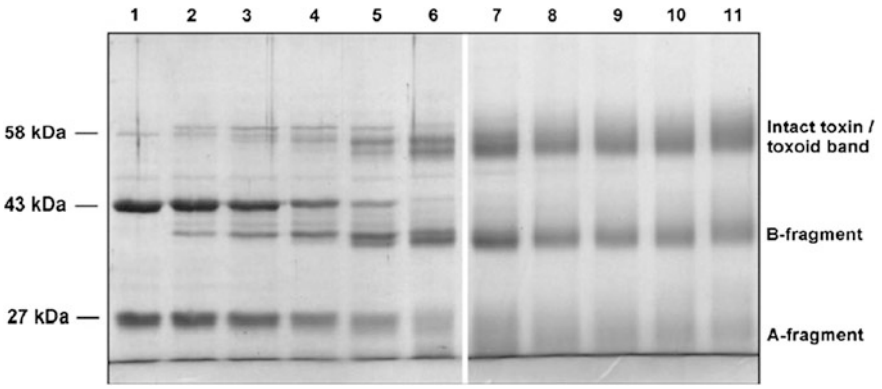


Fig. 6.2 SDS-PAGE of diphtheria toxin and toxoid. *Lane 1* Dipththeria toxin, *Lanes 2–11* Experimental toxoids prepared with increasing formaldehyde concentrations. Reproduced with permission from Elsevier (Metz et al. 2003)

6.2.2.3 Toxicity of Diphtheria Toxin

L+ is the minimum amount of diphtheria toxin that, when mixed and injected with 1 IU of specific antitoxin, kills an animal with a defined weight in 4 days (WHO 1965).

Minimum lethal dose (MLD) refers to the minimal amount of toxin that, when inject into a 250-g guinea pig, will cause death on the 4th or 5th day. The best purified preparation of toxin contains about 25 MLD/μg protein (Pappenheimer 1984).

The cell toxicity effect of diphtheria toxin can also be measured in Vero cell culture by titration of toxin with a fixed amount of antitoxin.

In the Vero cell culture, Phenol red is added to the medium as pH indicator. When pH drops from alkaline to acidic, this indicator changes color from red to orange to yellow. In the presence of excess of toxin cells are rapidly killed and the medium remains red. In the wells with excess antitoxin, the Vero cells survive and secrete acid metabolites, which induce a visible change of color from red to yellow. The point of equivalence is determined by twofold titration of the crude toxin. Using a spectrophotometer, the equivalence point can be determined more accurately (Aggerback and Heron 1991).

6.2.2.4 Antigen Purity

Purity (or specific activity) is expressed as Lf/mg protein nitrogen and a minimum value of 1,500 Lf/mg protein nitrogen is the WHO requirement (WHO 2014b).

6.2.2.5 Specific Toxicity (Ph. Eur. 0443)

Five SHDs of vaccine are injected subcutaneously into each of five healthy guinea pigs, each weighing 250–350 gm, that has not previously been treated with any material that will interfere with the test. If within 42 days of the injection, any of the animals show signs or dies from diphtheria toxemia, the vaccine does not comply with the test. If more than one animal dies from nonspecific causes, the test can be repeated once; if more than one animal dies in the second test, the vaccine does not comply with the test.

If this in vivo method is used for validation during the production stage of the vaccine, it may not be necessary to test the product at the final stage.

6.2.2.6 Absence of Toxin and Irreversibility of Toxoid (Ph. Eur. 0443)

A solution of bulk purified toxoid containing 100 Lf/mL is prepared using the same buffer solution as for the final vaccine without adsorbent. The solution is divided into two equal parts; one is maintained at 2–8 °C, while the other is kept at 37 °C

Table 6.5 Comparison of three guidelines for diphtheria toxoid specific toxicity and irreversibility

	Ph. Eur. 0443	WHO (2014b)	US (NIH 1947)
Specific toxicity	Inject five single human doses into at least five guinea pigs and observe 42 days	A 3.4.4: For bulk purified toxoid use 1 mL containing at least 500 Lf for each of the five guinea pigs, observe 42 days	Inject subcutaneously ≥5 SHDs (≥2.0 mL) into guinea pigs and observe 30 days without evidence of diphtheria toxin poisoning
		Cell culture test system may be used	
		A.3.5.2.5: For final bulk, use the same procedure as Ph. Eur.	
Absence of toxin and irreversibility of toxoid	Prepare diphtheria toxoid at 100 Lf/mL and incubate for 6 weeks at 2–8 °C or at 37 °C. Study toxicity in Vero cell, with and without antitoxin	A3.4.5: Dilute diphtheria toxoid to the same concentration as final bulk; incubate for 6 weeks at 2–8 °C or at 37 °C. Inject 10 SHDs (5 mL) to each of the five guinea pigs and observe 42 days. Only required for purified toxoid bulk but not for final bulk	Not described
		Intradermal test in guinea pigs and cell culture toxicity test are also considered suitable	

for 6 weeks. A Vero cell cytotoxicity assay is used to detect active diphtheria toxin in the presence and absence of diphtheria antitoxin. The toxoid sample fails the irreversibility test if toxicity neutralizable by antitoxin is present in either sample.

The guidelines vary slightly as outlined in Table 6.5.

6.2.2.7 Potency for Adsorbed Diphtheria Toxoid (Ph. Eur. 2.7.6)

This section describes potency method for DT per Ph. Eur. 2.7.6.

The potency is determined by administration of the vaccine to guinea pigs, followed either by challenge with diphtheria toxin (method A or B) or by determination of the titer of antibodies against diphtheria toxin or toxoid in the sera of guinea pigs (method C). The potency of the vaccine is calculated by comparison with a reference preparation, calculated in International Units.

Method A or B is used during development of a vaccines or whenever revalidation is needed following a significant change in the manufacturing process. In the interest of animal welfare, method C is used whenever possible.

Method A: Intradermal challenge test in guinea pigs
At least six groups of guinea pigs are injected subcutaneously with 1.0 mL of graded doses of test vaccine and reference preparation. The dilution should not be more than 2.5-fold from each other and the intermediate dilution should result in an intradermal score of approximately 3 (3 of the 6 challenge sites free from reactions) when 1.0 mL dose is used. After 28 days, shave both flanks of each guinea pig and inject 0.2 mL of each of the six toxin dilutions (containing 0.0512, 0.0128, 0.0032, 0.0008, 0.0002 and 0.00005 Lf) intradermally into six separate sites on each of the vaccinated animals in such a way as to minimize interference between adjacent sites. Examine all injection sites 48 h after and record incidence of specific diphtheria erythema. Record also the number of sites free from such reactions as the intradermal challenge score. Obtain relative potency by parallel-line quantitative analysis using skin test scores to calculate dose-response curve.

Method B: Lethal challenge test in guinea pigs
Groups of guinea pigs are immunized with test samples and reference vaccine dilutions for 28 days and challenged subcutaneously with 1.0 mL of 100 LD_{50} of toxin. Count the number of survived animal after 4 days. Calculate relative potency using statistical methods based on animal survival.

Method C: Inject subcutaneously to each guinea pig 1 ml of the diluted vaccine. Take a blood sample from each vaccinated and control animals 35–42 days later. Determine antibody titer by a sandwich ELISA or Vero cell assay.

The Vero cell assay relied on metabolic inhibition (method 1) or on cytotoxicity (method 2) as the endpoint and the cells are inspected microscopically or visually.

Method 1: The diphtheria toxin causes a cytopathogenic effect on Vero cells leading to cellular lysis. Antibody directed against diphtheria toxin may inhibit this effect. Consequently, the potency of a diphtheria vaccine may be indirectly determined with the help of this cell culture system if different serum dilution from immunized animals is cultured with a constant toxin concentration.

Method 2: Thiazolyl blue MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is reduced to a blue/black formazan product by the mitochondrial dehydrogenase of viable cells, and thus serves as a quantitative measure of living cells present, indicating when toxin has been neutralized by the antitoxin.

The Ph. Eur. challenge model in guinea pigs was used in establishing the 4th International Reference Standard for DT Adsorbed with a unitage of 213 IU/ampoule by the WHO Expert Committee on Biological Standardization in October 2009 (WHO 2014b). Additional collaboration study was performed using guinea pigs and mouse serological assays. Results suggest that Vero cell assay may be suitable for future replacement standards (Stickings et al. 2010) (Table 6.6).

Table 6.6 Summary of diphtheria potency assays from different guidelines

EP (Ph. Eur. 2.7.6)	WHO (2014b and 2013b)	USA (NIH 1947)
Immunize groups of guinea pigs with test and reference vaccines. At 28 days, challenge the animals with diphtheria toxins lethally (4-day read) or by skin test (2-day read). Alternatively, at 35–42 days, test sera by ELISA or by its ability to neutralize diphtheria toxin inhibition of Vero cell culture. Compare results with reference standard calibrated in international units	A.3.5.2.6: Same principle as EP. In addition to guinea pig challenge assays, WHO also allows immunization in mice with antibody levels titrated by toxin neutralization tests such as Vero cell assay (mice are not sensitive to diphtheria toxin challenge).	Immunize at least four guinea pigs with no more than one-half of the total human immunizing dose of the test vaccine and collect the immune serum 4 weeks later. A defined dilution of the immune serum is combined with a standardized quantity of diphtheria toxin and then injected into two naïve guinea pigs. Protection of test animals equates to toxoid potency. The L+ dose of the toxin is confirmed in the death of at least two control guinea pigs
	Potency used in immunization of children should be ≥30 IU/single human dose	Minimum requirement is ≥ 2 antitoxin units/mL for the pediatric dose

6.2.3 Acellular Pertussis Vaccine

Pertussis vaccine is used to prevent a childhood disease, whooping cough, caused by infection with *B. pertussis*. The first generation of the vaccine is the whole-cell pertussis vaccine, which contains inactivated bacteria (see Chap. 5) and is still used in most parts of the world. In higher-income countries, the whole-cell pertussis vaccine has been replaced with the less reactogenic aP vaccine.

The licensed aP vaccine contains up to five purified pertussis antigens, namely, pertussis toxoid, filamentous hemagglutinin (FHA), pertactin (69 kDa protein), and fimbrial agglutinogens 2 and 3. Pertussis toxoid is the essential component of the vaccine and a single-component pertussis toxoid vaccine is used to demonstrate the testing principle and strategy in this chapter, even though there is still debate on what components are required for protection (Tondella et al. 2009; Poolman and O Hallander 2007). The multiple component pertussis vaccine is discussed further in Sect. 6.2.3.13.

The production of the single-component aP vaccine involves the following steps:

1. Fermentation of *B. pertussis*
2. Purification of PT by affinity columns (Sekura et al. 1983)
3. Detoxification of PT by hydrogen peroxide treatment in the presence of ferric sulfate and EDTA (Sekura 1988).

Testing of PT, hydrogen peroxide inactivated pertussis toxoid and adsorbed toxoid (final bulk) is summarized in Table 6.7.

Table 6.7 Testing of a single-component acellular pertussis vaccine

Stages	General test	Product specific test
Purified toxin	Protein	Purity and characterization (SDS-PAGE, Western blot, and reverse-phase HPLC)
		Process-related impurities (blue dye and fetuin)
		CHO toxicity[a]
		ADP-ribosylation
		Hemagglutination (HA)
Unadsorbed toxoid	Protein	Inactivation kinetics
	UV spectrum	Residual HA
	Endotoxin (LAL)	Residual CHO toxicity[a]
		SDS-PAGE
		ADP-ribosylation
		Residual inactivation agent (hydrogen peroxide)
Adsorbed toxoid	Sterility	Histamine sensitization[a]
	Endotoxin	Irreversibility of toxoid
	Aluminum	Potency
	Thimerosal (if applicable)	% Adsorption
	pH	

[a] The step-by-step procedure is included in a WHO publication (WHO 2013b) for whole-cell pertussis vaccine, which is also applicable to acellular vaccine

6.2.3.1 Purity of Pertussis Toxin and Toxoid by SDS-PAGE

Pertussis toxin is the major virulence factor of *B. pertussis*. After being purified by Affi-Gel blue and fetuin-Sepharose 4 B affinity columns, its purity was verified by SDS-PAGE shown in Fig. 6.3 (Sekura et al. 1983).

Once PT is chemically detoxified by hydrogen peroxide in the presence of Fe^{+3} and EDTA (Sekura 1988), the structure becomes heterogeneous and characterization became difficult. Corbel et al. (1999) showed that the bands on the SDS-PAGE became fussy and broadened for the detoxified protein (Fig. 6.4).

6.2.3.2 Product-Related Impurities

The contaminant level of pertussis antigens had to be established as part of the product characterization, because trace amount of some pertussis antigens may play a role in protection or toxicity. Using Western blot and monoclonal antibodies specific to FHA, adenylate cyclase, pertactin, and fimbriae, the assay sensitivities are defined and the purified toxin is tested. The other potential toxic contaminants include heat labile (dermonecrotic) toxin and tracheal cytotoxin. Heat labile toxin can be detected by observing dermonecrotic activity after subcutaneous injection of

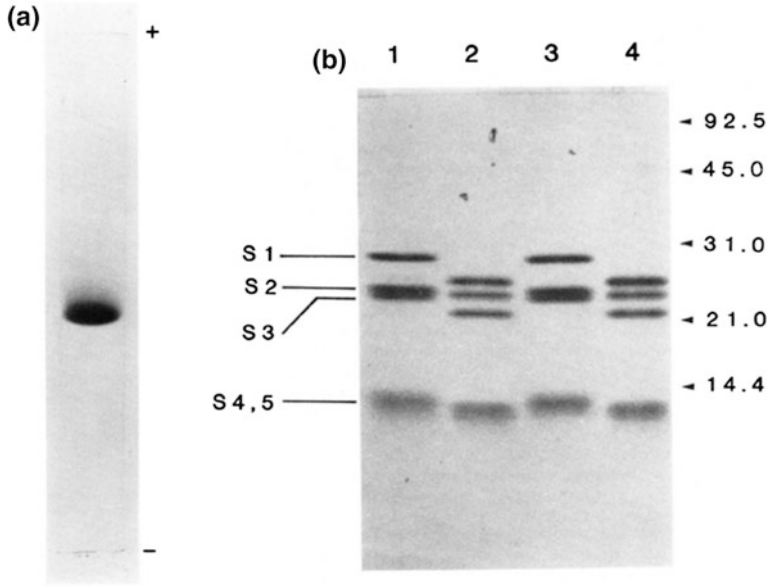


Fig. 6.3 Panel **a** Purified pertussis toxin on 5 % native gel. Panel **b** Pertussis toxin on 15 % SDS-PAGE. Reproduced with permission from The American Society for Biochemistry and molecular biology (Sekura et al. 1983). *Lanes 1* and *3* reducing samples, *Lanes 2* and *4* nonreducing samples

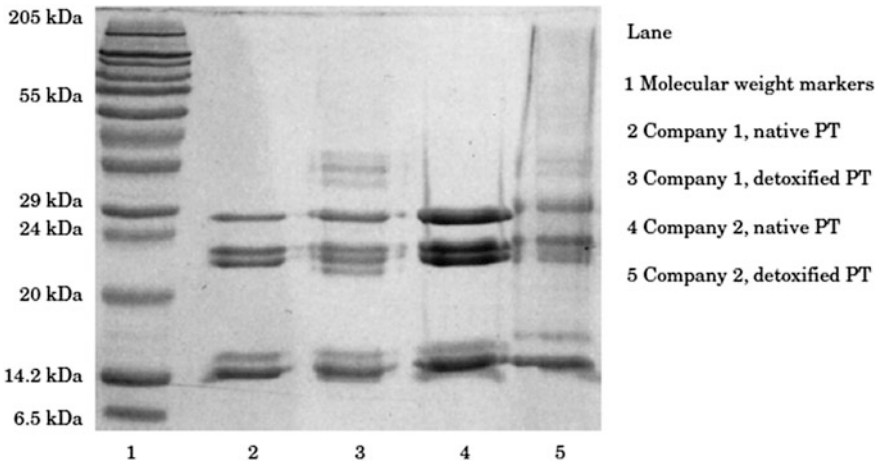


Fig. 6.4 Comparison of native and detoxified pertussis toxin on a 15 % SDS-PAGE with Coomassie blue stain. Reproduced with permission from Elsevier (Corbel et al. 1999)

the test material into the nuchal area of suckling mice (WHO 2013b). Tracheal cytotoxin, a disaccharide-tetrapeptide with molecular weight of 921 Da, can be tested by HPLC (Cookson et al. 1989).

The limits for pertussis impurities are defined in the EP (Ph. Eur. 1356, pertussis vaccine, acellular, component, adsorbed). The limit of adenylate cyclase is 500 ng per dose, for tracheal cytotoxin, no more than 2 pmol per dose and for dermonecrotic toxin, three unweaned mice each injected with 0.1 mL, containing the equivalent amount of antigenic fraction or component present in one dose, should show no dermonecrotic reaction within 48 h.

The active ingredient of one commercial aP bulk vaccine is a hydrogen peroxide inactivated pertussis toxoid at 40 μg per dose. The pertussis antigen contamination level verification was a critical part of the comparability study when postlicensure process changes were submitted for regulatory approval.

6.2.3.3 Process-Related Impurities

Assays for residual levels of ligands used in affinity purification (e.g., Blue dye and fetuin) are included in the impurity profile. Cibacron Blue F3G-A absorbs at 610 nm with an extinction coefficient of 13,600 M^{-1} cm^{-1} (Lowe and Pearson 1984) and a limit assay can be established based on UV/Visible absorbance scan. Residual fetuin can be tested by a suitable immunological method (ELISA or rocket electrophoresis) using purified rabbit anti-fetuin antibody.

6.2.3.4 HPLC Characterization of Pertussis Toxin

PT contains 6 subunits with an A-B structure and molecular weight of 117 kDa. The A component is composed of a single S1 subunit with ADP-ribosyl transferase activity. The B oligomer, consists of single S2, S4, and S5 subunits and two S3 subunits, mediates receptor binding and intracellular translocation (Tamura et al. 1982). Reverse-phase HPLC has been used to separate the subunits and LC-MS/MS used in the subunit sequence determination (Tummala et al. 2008). The subunits 1, 2, and 5 of PT were observed to undergo oxidation under normal storage conditions as ammonium sulfate suspension at 2–8 °C (Fig. 6.5).

After detoxification with hydrogen peroxide, there was no clear separation of toxoid subunits in the reverse-phase HPLC. Two-dimensional liquid chromatography-tandem mass spectrometry, ultra performance liquid chromatography/Mass spectrometry, and capillary liquid chromatography/MALDI tandem mass spectrometry were used to analyze the tryptic digest of pertussis toxoid. Hydrogen peroxide treatment in the presence of Fe^{+3} modified nearly all the methionine and tryptophan residues and a large percentage of cysteine, aspartic acid, and histidine. Some tyrosine, Phenylalanine, proline, and glutamine were also modified (Tummala et al. 2010, 2013). This type of product characterization is laborious and unpractical for routine analysis. Instead, loss of biological activities (histamine sensitization, CHO toxicity, and hemagglutination) is measured routinely following detoxification.

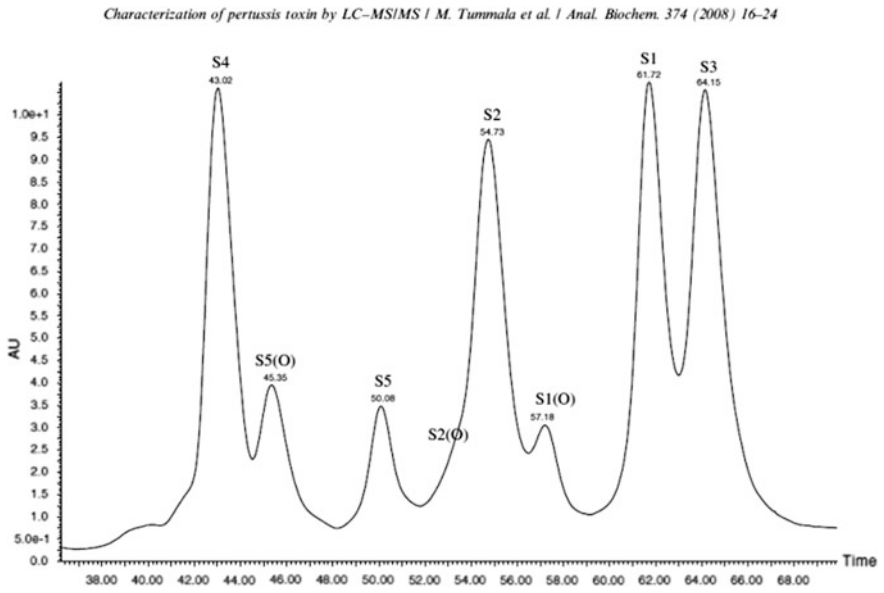


Fig. 6.5 Reverse-phase HPLC separation of pertussis toxin. Reproduced with permission from Elsevier (Tummala et al. 2008)

Of the components used in the formulation of multicomponent aP vaccines, only PT has significant intrinsic toxicity. The other components, FHA, pertactin, Filbriae 2 and 3 have not been shown to exert toxic effects. The safety testing is implemented to regulate the residual PT activity in the product. There is no general agreement on choice of assay or acceptable limits for residual toxicity.

6.2.3.5 Hemagglutination Assay for Toxin and Unadsorbed Toxoid

PT causes goose red blood cell to agglutinate; it is a rapid assay used for process monitoring during PT purification (Sekura et al. 1983). Hemagglutination activity requires only the B subunit of PT, which provides the binding function. Detoxification of PT with hydrogen peroxide in the presence of EDTA and Fe^{+3} brings hemagglutination activity down to <1 % (Sekura 1988).

6.2.3.6 CHO Cell Toxicity for Toxin and Unadsorbed Toxoid

Exposing CHO cells in culture to PT results in a clustered growth pattern (Hewlett et al. 1983). The clustering of CHO cells correlates with ADP-ribosylation of a 41-kDa protein in CHO cells and thus a true measure of PT activity for both A and B subunits (Burns et al. 1987). The CHO cell assay has been used as an in vitro test

to detect residual active PT in the vaccine. It has been suggested that toxoid aggregation could give false-negative results with formaldehyde-inactivated vaccines (Kataoka et al. 2002). Moreover, adjuvant can cause CHO cell death (Corbel and Xing 2004) and a porous cell culture insert is used to prevent vaccine adjuvant from directly contacting the CHO cells (Isbrucker et al. 2014).

6.2.3.7 ADP-Ribosylation

The S1 subunit of PT harbors ADP-ribosyl transferase activity with a GTP-binding protein (G protein) being the natural substrate; this enzymatic assay can be used to quantify PT and monitor the extent of detoxification. A fluorescent-tagged synthetic peptide homologous to the carboxy-terminal 20 amino acid sequence of the alpha subunit of G protein was used as the substrate for enzyme assay. The tagged peptide and the ADP-ribosylated product were separated by HPLC with fluorescent detection (Cyr et al. 2001). The assay could detect as little as 2 ng toxin and worked well with adsorbed product to detect the residual toxin activity (Yuen et al. 2002).

6.2.3.8 Kinetics of Inactivation

Kinetics of inactivation should be established as part of the manufacturing process validation (WHO 1998). Hemagglutination assay and ADP-ribosyl transferase assay have been used to establish the inactivation kinetics to demonstrate the consistency of inactivation (Sekura 1988).

6.2.3.9 Histamine Sensitization Test (HIST) and Irreversibility of Pertussis Toxoid (Ph. Eur. 2.6.33)

HIST is the official Pharmacopeia test used for detecting residual PT activity in the adsorbed vaccine. Groups of at least 10 mice, of suitable strain, age, and weight, are injected intraperitoneally with between 1 and 2 human doses of the adsorbed test vaccine. The first group receives vaccine stored at 2–8 °C (test for residual PT); the second group receives vaccine incubated at 37 °C for 4 weeks (test for irreversibility of toxoid), and the third group receives diluent (negative control).

In addition, a positive control group of mice is injected with an equivalent volume of reference PT preparation at a dose that has been defined in the validation stage as demonstrating the assay sensitivity.

If a reference group of mice is used, it may be injected with a reference PT preparation at a dose previously set as the allowable upper limit of PT in the product, according to historical safety data. Alternatively, a reference vaccine with established clinical safety may be used instead of the reference toxin preparation.

Five days later, animals are challenged intraperitoneally with histamine solution (2 mg histamine base in ≤0.5 mL) and the number of mice dying within 24 h is

recorded. If a reference group is included, the vaccine complies with the test for residual PT if the percentage of deaths in the first group is not greater than that in the reference group. The vaccine complies with the test for irreversibility of pertussis toxoid if the second group also complies with these criteria. If no reference group is included and no animal dies, the preparation complies with the test.

The mice strain is suitable if its LD_{50} is between 6 and 50 IU. The test is not valid if one or more mice in the negative control group die following histamine challenge and the histamine sensitivity does not meet the defined limit (e.g., at least 30 % of the mice die in the positive control group).

The aP vaccine licensed in the United States follows a slightly different protocol and acceptance criteria. The USA, WHO and EP procedures together with HIST theory and history were reviewed by Arciniega et al. (2011). The key differences between the three guidelines are summarized in Table 6.8.

The assay procedure varies from one laboratory to the other. In an international collaborative study involving six laboratories, HSD_{50} values obtained in this study differ significantly between laboratories (Xing et al. 2002).

Table 6.8 Comparison of HIST protocol between USA, WHO, and Ph. Eur.

	Ph. Eur. 2.6.33	WHO (2013a) Appendix 3	USA (Arciniega et al. 2011)
Mice	≥10 mice	Appropriate number	20 mice 15–20 gm 4–5 weeks old, female
Amount injected	1–2 human doses intraperitoneally	1–2 SHDs intraperitoneally	One human dose in 0.5 mL intraperitoneally
Histamine formula	2 mg histamine base/ ≤ 0.5 mL	1–2 mg histamine base	1 mg histamine base/ 0.5 mL
Challenge date after injection	5 days	4–5 days	5 days
HSD_{50}	6–50 IU	Not specified	10–100 ng
Allowed death in control	0 (No control mouse should die for the assay to be valid)	≤5 %	≤10 %
Allowed death in sample	0 (no test mouse should die for the assay to pass)	PT activity ≤ clinical lots	≤10 %
Retest criteria after initial failure	Could be repeated if one mouse dies	Two consecutive assays must pass	Retest two consecutive times and both must pass
	The assay passes if total death in all valid assays ≤ 5 %		

In the subsequent international collaboration study involving ten laboratories for the aP and the combination vaccines, use of a validated procedure and inclusion of a common reference standard were shown to greatly improve HIST between laboratory agreements and reduce repeat testing. A potency of 7,500 IU/vial was assigned to the Ph. Eur. Biological reference preparation for PT (Xing et al. 2010).

Concerns about the reliability of the lethal endpoint have been reported (Xing et al. 2010) and alternative endpoint measurement has been proposed. Jensen et al. (2012) developed a dermal temperature-based HIST assay and estimated a sensitivity of 5 ng PT per human dose of pertussis vaccine using this modified procedure. In this method, the change in body temperature after histamine challenge is measured and sensitized mice show a sharp decrease in body temperature following histamine challenge. The dermal temperature-based method, which has been incorporated to the HIST procedure used routinely in Japan, has been shown to be as sensitive as the method based on rectal temperature measurement (Ochiai et al. 2007). The use of a histamine-sensitization test based on body temperature measurements as endpoints in mice has been approved in Ph. Eur. 2.6.33.

HIST is difficult to standardize, its precise mechanism is unknown, the amount of PT that is unsafe for human is also unknown, and therefore it was regarded as a priority for replacement by in vitro methods. A new in vitro test system was shown to be a potential alternative to the current HIST. The in vitro test system is based on two assays, the HPLC assay measuring ADP-ribosyl transferase activity of the S1 subunit of PT described in Sect. 6.2.3.7 and a fetuin binding ELISA based on the activity of the B protomer. A mathematical formula showed a good agreement with HIST results, based on dermal temperature reduction after histamine challenge. A regression factor should be established for each type of vaccine (Yuen et al. 2010). Arciniega et al. (2011) have expressed concerns about how an acceptance limit could be set for residual PT activity using the dual in vitro assay. Subsequent international collaborative study did not establish a direct correlation between the in vitro assays and the in vivo HIST (Xing et al. 2012). Several workshops have been held to discuss alternatives to the histamine sensitization test and a working group was established for ongoing discussions (Bache et al. 2012; Isbrucker et al. 2014).

6.2.3.10 Potency

According to Ph. Eur. 2.7.16, two serological potency assays are available for aP vaccine.

Method A: Serology in mice

Six groups of 5-week-old healthy mice are injected intraperitoneally or subcutaneously with 0.5 mL of three dilutions of either the test vaccine or a reference vaccine (for example, a batch shown to be effective in clinical trials, or a batch representative thereof). The mice are bled 4–5 weeks later and antibody titer is determined by ELISA using plates coated with each pertussis antigen (if the test is

for multicomponent aP vaccine). Antibody titer is calculated in relative terms to a reference serum.

Method B: Serology in guinea pigs

Immunize at least six groups (three dilutions each for test and reference vaccines) of guinea pigs with 1.0 mL of vaccine subcutaneously. Bleed the animal 35–42 days later. Determine antibody titer by ELSA coated with purified antigen.

No completely satisfactory method is available for monitoring pertussis potency. Immunogenicity assays are useful for checking consistency in comparison to a clinical trial lot, but do not necessary correlate with protection.

A modified intracerebral challenge assay (MICA, modified Kendrick test) has been used in Japan, Korea, and China as the potency assay for release with a specification of ≥4 unit/dose; vaccines regulated using this approach have been shown to be effective in controlling pertussis (Tondella et al. 2009). MICA appears to be a suitable model to assess the potency of aP vaccines of different antigen compositions (Knezevic et al. 2008), but it is also a consistency test whose correlation to protection has not been demonstrated.

The modified Kendrick test is described here. Groups of mice are injected intraperitoneally with 0.5 mL serial dilutions of the reference and the test vaccines. At 3 weeks after immunization, mice are challenged intracerebrally with a *B. pertussis* suspension prepared from a 20–24 h culture grown on Bordet-Gengou agar or other suitable medium. The bacteria suspension is adjusted in such a way that each challenge dose of not more than 0.03 mL contains 100–1,000 times of LD_{50}. The mice are observed for lethal effects over the next 14 days. The potency is estimated in terms of international units by parallel-line assay. The modification from the original Kendrick assay includes a longer interval between immunization and challenge (3 weeks instead of 2 weeks) and using a special mouse strain (Corbel and Xing 2004).

In an international collaborative study initiated in September 2006, JNIH-3 aP vaccine reference was found to give similar dose-response lines to a variety of aP vaccines and DTaP formulations, irrespective of the differences in aP components. The WHO working group established it as the First International Standard for aP vaccine in MICA. (WHO 2013a) JNIH-3 contains lyophilized PT and FHA adsorbed onto aluminum phosphate with an assignment of 34 IU per ampoule (Gaines-Das et al. 2009).

6.2.3.11 Percentage Adsorption

For low or moderate level of adsorption, there is sufficient amount of antigen in the supernatant (unadsorbed material) to be determined by a protein assay. For high level of adsorption, a more sensitive immunological assay is used to determine the unadsorbed antigen in the supernatant.

6.2.3.12 Residual Inactivation Agent (Hydrogen Peroxide)

Residual hydrogen peroxide is tested with Quantofix Peroxide test sticks purchased from Sigma Aldrich. It is a limit assay with 10 ppm sensitivity (10 µg/mL solution).

6.2.3.13 Process and Testing of Multicomponent Acellular Pertussis Vaccine

Two approaches have been followed to produce multicomponent aP vaccines. In the first approach, vaccine components have been co-purified and the purified material is detoxified with formaldehyde. The second approach is to purify each component individually, detoxify when required and blend to produce the vaccine bulk (Corbel and Xing 2004). For example, DAPTACEL, a DTaP combination vaccine licensed in the USA, contains five pertussis antigens. PT, FHA, and pertactin are purified separately from *B. pertussis* culture supernatant and fimbrial agglutinogens 2 and 3 are co-purified from the bacteria cells. PT is detoxified with glutaraldehyde and FHA is treated with formaldehyde. The individual antigens are adsorbed separately onto aluminum phosphate and combined.

Due to the toxoiding process, these vaccines do not have a defined chemical composition. To monitor consistency, biological testing is required. Ph. Eur. 1595 describes testing for adsorbed, co-purified aP vaccine and Ph. Eur. 1356 describes testing for adsorbed, individually prepared and purified aP vaccine. Most of the methods have been discussed earlier in Sect. 6.2.3. The testing not mentioned in subsections of Sect. 6.2.3 is outlined below.

a. Specific properties of the additional components.

(1) FHA: Hemagglutination and inhibition by specific antibody. PT and FHA both have hemagglutinating activities and they are discerned with FHA specific antibody.

(2) Pertactin and fimbrial agglutinogens 2 and 3: reactivity with specific antibody.

(3) Pertussis toxoid: The toxoid induces in animals the production of antibodies capable of inhibiting all PT activities. For example, CHO neutralization assay demonstrates CHO activity of PT neutralized by the antibody produced from pertussis toxoid.

(4) For individually purified antigen, the purified components must be characterized separately by physicochemical, immunological, or biological assays before detoxification.

b. Antigen content: Immunochemical methods are used to determine quantitative antigen composition. The ratio of each antigen to total protein is within the limits established for the product.

c. Potency: Antibodies to each individual pertussis antigen are measured and compared to the reference vaccine (Ph. Eur. 2.7.16). For example, potency of DAPTACEL is determined by the antibody response of immunized mice to detoxified PT, FHA, pertactin, and fimbrial agglutinogens as measured by ELISA.
d. Final container identity testing: Specific antisera to the components are used.

6.2.4 Testing of DTaP Combination

6.2.4.1 Percent Adsorption

The supernatant of the adsorbed vaccine is tested for D, T, or aP component using suitable immunological methods. In the DTaP vaccine reported by Bergfors et al. (2003), the manufacturer used Rocket electrophoresis to determine % adsorption for D and T toxoids and used ELISA for aP adsorption. Coombes et al. (2009) developed a capture ELISA and a direct ELISA to measure percent adsorption for Diphtheria component in combination vaccines. Another capture ELISA for percent adsorption of tetanus toxoid in combination vaccines was reported by Coombes et al. (2012). In addition, the step-by-step procedure for D and T capture ELISA was provided by WHO (2013b).

6.2.4.2 Identity

Each component is identified by a suitable immunological method. Although Ph. Eur. 1931 and WHO guidelines (1990, 2013b) describe dissolving adjuvant or eluting toxoid, the ELISA identity test can work without dissolving adjuvant or eluting toxoid.

6.2.4.3 General Safety (Innocuity)

Half a human dose (<1 mL) is intraperitoneally injected into each of 5 mice (17–22 gm) and at least one human dose (<1 mL) is injected into each of 2 guinea pigs (250–350 gm); all animals should survive at least 7 days without showing significant signs of toxicity (WHO 1990).

The USA procedure uses 0.5 mL vaccine for each of the 2 mice and 5.0 mL for each of the 2 guinea pigs (21CFR610.11).

6.2.4.4 Specific Toxicity (Ph. Eur. 1931)

For Tetanus, see Sect. 6.2.1.7. Inject subcutaneously at least 5 SHDs to each of the 5 guinea pigs and observe 21 days for paralysis.

For Diphtheria, see Sect. 6.2.2.5. Inject subcutaneously at least 5 SHDs to each of the 5 guinea pigs and observe 42 days for diphtheria toxemia.

The same animals are used for both DT and TT tests and are observed for 6 weeks to cover the observation period specified for DT.

For pertussis component, see Sect. 6.2.3.9 for histamine sensitization.

6.2.4.5 Aluminum Adjuvant

The aluminum adjuvant in the vaccine is first digested with a mixture of nitric and sulfuric acid at high temperature and then measured by atomic absorption (May et al. 1984) or by titration (Ph. Eur. 2.5.13).

6.2.4.6 Thimerosal

Thimerosal is determined by a spectrophotometric assay (Shrivastaw and Singh 1995b).

6.2.4.7 Formaldehyde

A colorimetric reaction was described by Nash (1953), which depends on the synthesis of diacetyldihydrolutidine from acetylacetone and formaldehyde in the presence of excess ammonium salt (Hantzsch reagent). The diacetyldihydrolutidine formed is yellowish-green and highly absorptive at 412 nm. A standard curve with formaldehyde can be established as a quantitative assay. Ph. Eur. 2.4.18 (method A) described it as a limit assay by visually comparing vaccine dilution with 20 μg/mL formaldehyde solution after the reaction. The limit established by WHO (1990) was 0.2 gm/L.

Another formaldehyde colorimetric assay was described by Shrivastaw and Singh (1995a). In this system, formaldehyde reacts with phenyl hydrazine/potassium ferricyanide and chloroform in the presence of methanol to form a colored complex. The adsorbed product is tested without having to remove the adjuvant.

6.2.4.8 Endotoxin (Ph. Eur. 2.6.14)

To quantify endotoxins in the vaccine samples, amoebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*) is used. There are three techniques for this test: the gel-clot technique, which is based on gel

formation; the turbidimetric technique, based on the development of turbidity after cleavage of an endogenous substrate; and the chromogenic technique, based on the development of color after cleavage of a synthetic peptide-chromogen complex.

6.2.4.9 PH, Sterility, Appearance

These compendial methods are not described here.

6.2.4.10 Potency

The animal potency tests currently required by WHO, the EP, and FDA differ. Despite these differences, the potency tests have been adequate to ensure sufficient immunogenic activity of the vaccines to induce protective immunity in target populations.

It is recommended to perform potency testing after combination of the individual diphtheria and tetanus toxoid components in Diphtheria and Tetanus Toxoid vaccines for pediatric use (Federal register 2005)

For TT potency, see Sect. 6.2.1.8 and for DT potency, Sect. 6.2.2.7

The US method allows D and T potency to be tested in the same group of guinea pigs (NIH 1953; Keller 2011).

For aP potency see Sect. 6.2.3.10.

6.3 MenB OMV: Outer Membrane Vesicles

The epidemiology of endemic meningococcal disease in many developed countries is currently caused by serogroup B meningococcus, responsible for septicemia, meningitis, and killing children and young adults in few hours (Feavers et al. 2012). In the past, the vaccine approach to group B meningococci has largely focused on OMPs (Outer Membrane Proteins) as part of OMVs (Outer Membrane Vesicles) that were prepared by detergent extraction from the bacterium. Antigen presentation in the vesicle structure has the main advantage to preserve the physicochemical organization of the OMPs and consequently their immunogenicity (Holst et al. 2009).

Until recently, OMV vaccines were the only formulations licensed against serogroup B meningococcal disease. Efficacy has been demonstrated by their ability to control clonal epidemics, and strain-specific vaccines, such as MenBvac (NIPH) and MeNZB (Chiron, later Novartis Vaccines) have been successfully used to combat outbreaks in Norway and New Zealand, in the 1990s and early 2000s, respectively (Fredriksen et al. 1991; Holst et al. 2005).

Unfortunately, this approach is very successful in providing protection against homologous strains but not against heterologous strains due to sequence and antigenic variability of the main antigenic components. During the last decade, a

new generation of vaccines has been developed with the aim of providing broader coverage and the first multivalent serogroup B (4CMenB) vaccine named Bexsero® has been recently licensed for human use in Europe starting from 2 months of age. The vaccine includes the OMV MeNZB vaccine as one of the components (Serruto et al. 2012).

The concentrated OMV antigen is a stable colloidal suspension that consists of small membranous spherical vesicles in which the native complex antigen composition of the subcapsular cell surface of *Neisseria meningitidis* serogroup B is highly conserved. As a result, the OMV particles contain the most abundant surface-exposed proteins, (PorA and PorB are the main components), several less abundant proteins, and low amounts of LPS, embedded in the vesicles as presented in Fig. 6.6. Additional components such as phospholipids and CPS (Capsular Poly Saccharides) are also part of the vesicle structure.

The OMV particles are produced by fermentation of the appropriate *N. meningitides* strain, followed by concentration and bacterial inactivation with DOC (deoxycholate), also mediating OMV formation. The OMVs are then separated, sized, and purified by centrifugation and sonication steps, followed by a final filtration step using a 0.22-μm membrane (Frasch et al. 2001).

Quality and consistency of OMV concentrated bulk production is monitored by adherence to release the specifications as listed in Table 6.9.

Due to the complex structure and presence of several antigenic components, all with different highly informational structures, the OMV particles present many analytical challenges. Dedicated analytical methods are required to characterize the OMV particles on a molecular level and to monitor key components of these complex structures.

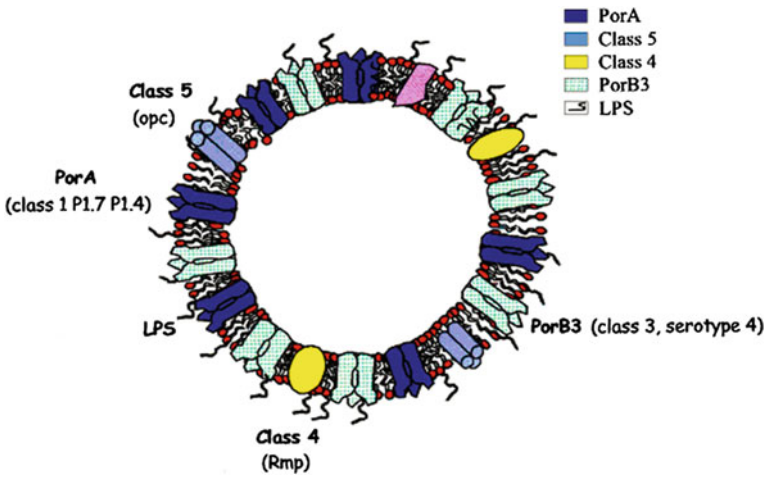


Fig. 6.6 Schematic model of OMV (strain NZ 98/254 -MeNZB): OMVs are portions of the outer membrane of the MenB, extracted with detergent in the form of vesicles [Figure adapted from Holst et al. (2009) with permission]

Table 6.9 Example of OMV (as component of MeNZB) bulk release specifications

Critical quality attribute	Analytical method	Release specification	Stability indicating	Rationale
Identity	Western blot	Presence of class 1 (P1.4), class 3 (ST 4), class 5 (OPC), LPS 3, 7, 9	Yes	The test indicates positive reaction to antibodies directed against LPS and the most crucial protein antigens eliciting responses in humans (the most important is PorA or Class 1)
Protein content	Lowry	450–1,240 μg/ml	Yes	The test measures the total amount of proteins present in OMV, including the major immunogenic antigens PorA and PorB
Protein pattern	Coomassie SDS-PAGE	Purity: conform 70 kDa: 1–12 %; 80 kDa: 1–4 %; Class 1: 13–25 %; Class 3: 20–55 %; Class 4: 7–15 %; Class 5: 1–5 %; NspA: 1–7 %	Yes	The test measures by electrophoresis the presence and relative percentage of the main protein antigens. Moreover, it evaluates (as purity) their content respect total lane proteins
DOC/protein	Enzymatic reaction followed by colorimetric test	0.1–0.4 μg/μg	No	The test measures the presence of residual DOC impurity in the OMV bulk
LPS/protein	RP-HPLC	0.04–0.12 μg/μg	No	The test evaluates the LPS total amount in the OMV bulk by chromatographic determination of 3OH 12:0 fatty acid component of Lipid A
Endotoxin/protein	Kinetic chromogenic LAL	<20,000 IU/μg	Yes	Due to the presence of LPS in the vesicles extracted from the bacteria, the OMV purified

(continued)

Table 6.9 (continued)

Critical quality attribute	Analytical method	Release specification	Stability indicating	Rationale
				bulk presents some endotoxicity. The test evaluates the LPS bioavailability, influenced by Lipid A insertion in the vesicle bilayer
DNA/ protein	Fluorimetry	≤0,035 μg/μg	No	The test measures the presence of residual DNA impurity in the OMV bulk
pH	Potentiometry (Ph. Eur.)	7.0–8.3	Yes	The test measures the pH of the OMV bulk suspension
Sucrose %	Spectrophotometry	2.7–4.1 %	No	The test measures the quantity of sucrose, added to the OMV suspension as preservative
Sterility	Membrane filtration (Ph. Eur.)	Sterile	No	The test measures sterility of the OMV drug substance, required before vaccine formulation

Four quality attributes of the OMVs requiring specific analytical focus were explored by extended physicochemical characterization and are described in the subsequent sections. These analytics are aimed to antigen structural understanding and can be used as additional tools in case of comparability studies.

6.3.1 OMV (MenB NZ) Dimensions and Morphology

Chemical composition and quantity of the single constituents are not the only parameters sufficient for characterizing the OMVs as vaccine component. Vesicle dimensions, morphology, and homogeneity are important structural features that may influence immunogenicity and thus need to be controlled. One example of the importance of vesicle integrity is related to the LPS component of the OMV. LPS

endotoxicity is reduced when the Lipid A moiety is inserted into the vesicle lipid bilayer (Tsai et al. 1989). Consequently, evaluation of the OMV bulk vesicle integrity is important as it relates to pyrogenicity.

Here, we show three different and complementary analytical methods to study OMV size, morphology, and dimensional distribution: DLS (Dynamic Light Scattering), TEM (Transmission Electron Microscopy), and SE-HPLC (Size Exclusion Chromatography).

OMV dimensions are in the subvisible range (0.001–100 μm) and can be measured by in-batch DLS able to determine the vesicles average diameter and the population polydispersity in a fast and simple manner (Dahneke 1983).

DLS was applied to OMV bulk characterization and comparability studies between different production campaigns and during manufacturing development to evaluate in-process parameters of the sonication step necessary to improve material filterability and homogeneity.

The average vesicle diameter of OMV bulk production (determined from data on more than 40 lots) resulted between 97 and 154 nm. PdI (polydispersity Index, distribution confidence ratio related to Z-average measure), estimating the width of the distribution was typically determined to be in the range of 0.1 and 0.3 and indicated, as expected, presence of a multimodal distribution.

Figure 6.7 shows a representative DLS output dataset for OMV bulk. The upper panel reports the input parameters for the analysis. In the lower panel, a Gaussian

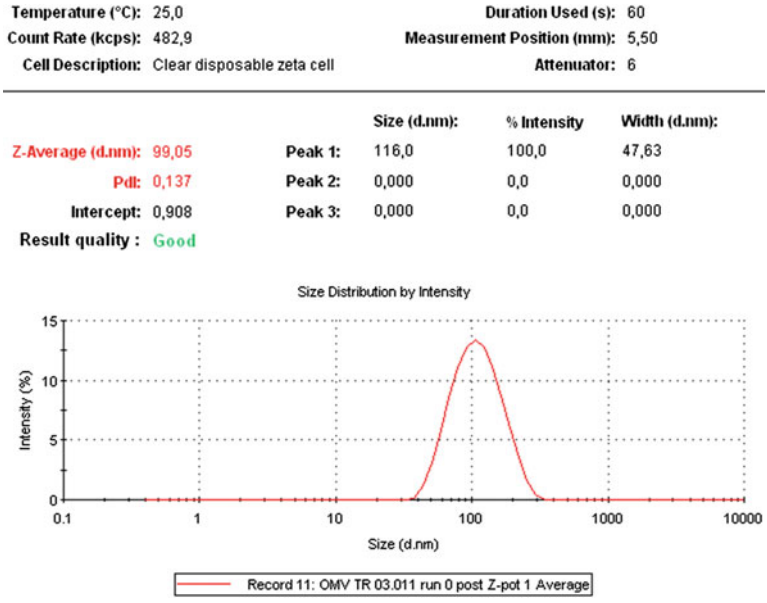


Fig. 6.7 Representative DLS output data for OMV bulk production. Highlighted in *red* are Z-average diameter (d.nm, average diameter in nanometers of the populations) and PdI (distribution confidence ratio related to Z-average measurement)

distribution is visible for the vesicles population with an average diameter (Z-average diameter (d.nm)) of 99 nm and a polydispersity index (PdI) of 0.14.

Dimensional data obtained by DLS were integrated and confirmed by use of complementary TEM analysis. TEM provides information on vesicle morphology and integrity. In addition, TEM can be used to localize antigens on the vesicle surface.

OMVs were analyzed by TEM in negative staining mode by using uracil acetate and 4 % glutaraldehyde. The negative staining TEM allowed examination of the vesicles' external surface by visualizing shape and integrity.

A set of 14 OMV concentrated bulk lots were analyzed. The vesicles consisted mainly of rounded shaped vesicles, with dimensions in the range of 50–200 nm (in agreement with DLS data), presenting light or dark tonalities, possibly connected to concavity/convexity. A representative OMV TEM picture is shown in Fig. 6.8.

The TEM images can be used to evaluate vesicle integrity. TEM images can also be applied to gain information on antigens localization on the OMV surface. By utilizing anti-LPS (3, 7, 9) antibodies and immunogold staining, LPS can be localized on the vesicle surface as small dark dots (Fig. 6.9).

SE-HPLC was applied to separate chromatographically the dimensional components of the OMV bulk solution to complement the DLS in-batch analysis. The results showed that SE-HPLC is able to discriminate up to 4–5-dimensional populations differing in size when applied to the OMV batches.

The chromatographic separation resulted not completely quantitative due to retention of liposomal vesicles by the column matrices tested. However, the

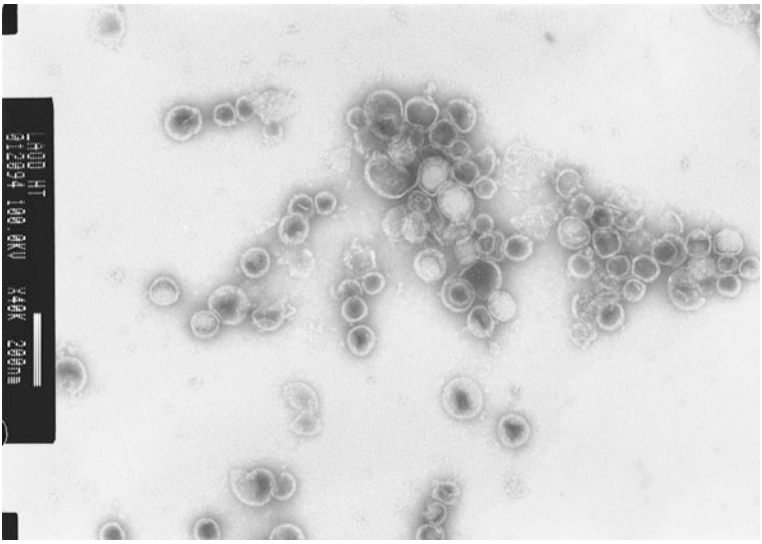


Fig. 6.8 Representative TEM image of OMV concentrated bulk. The *bar* on the *left* represents 200 nm

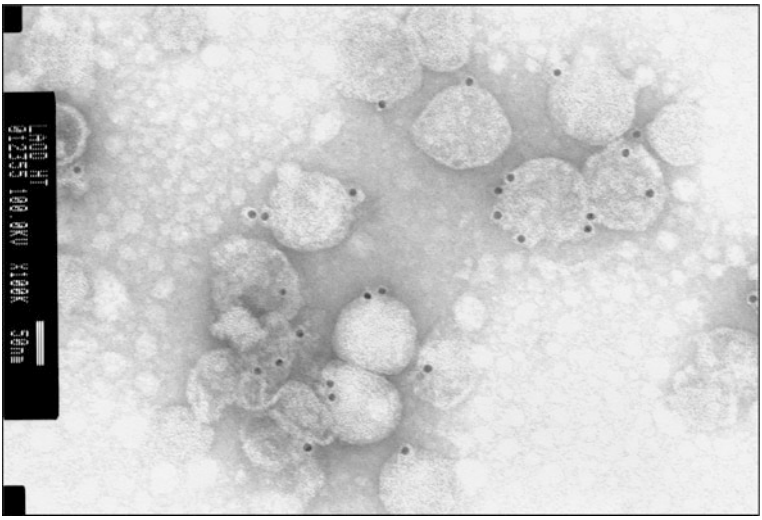


Fig. 6.9 Immunogold staining of LPS (3, 7, 9) on the OMV surface. The *bar* on the *left* represents 50 nm

SE-HPLC analysis remains a relevant complementary method to DLS for the investigation of vesicle degradation.

Figure 6.10 shows the chromatographic profiles of two OMV bulk lots produced at different times and analyzed by SE-HPLC. The individual peaks were fractionated and analyzed by DLS and TEM for characterization. Compared to the unfractioned sample (about 100 nm size average and 86 % integrity by visual TEM evaluation), the main peak in lot 1 (Fig. 6.10 upper panel, red frame) was shown to contain intact OMVs of about 100 nm size average and 97 % integrity and the second fraction (Fig. 6.10 upper panel, blue frame) smaller vesicles with 82 % integrity. In the third fraction (Fig. 6.10 upper panel, green frame), no intact vesicles were detected by TEM, suggesting presence of membrane fragments and cellular debris. Lot 2 (Fig. 6.10 lower panel) was found to be similar to the lot 1 but with minor differences in the vesicle populations of smaller size as well as in the membrane fragments/cellular debris content. In this case, the main peak (Fig. 6.10 lower panel, gray frame), was found to consist of a homodisperse population of vesicles of about 100 nm average diameter when analyzed by DLS.

The effect of physical and chemical stress on vesicle integrity, dimensions, morphology, and homogeneity was investigated to identify conditions that may induce the release of free LPS from the membrane. LPS forms stable spherical membrane structures with proteins and phospholipids where the toxic lipid part of LPS (Lipid A) is buried into the interior of the membrane. Approximately 4–8 % of LPS (calculated as LPS/protein ratio) remains as an integrated part of the vesicle (Lyngby et al. 2002). This structural organization is extremely important because the toxicity of LPS is substantially reduced when integrated in the OMV compared to free LPS in solution (Lyngby et al. 2002; Tsai et al. 1989).

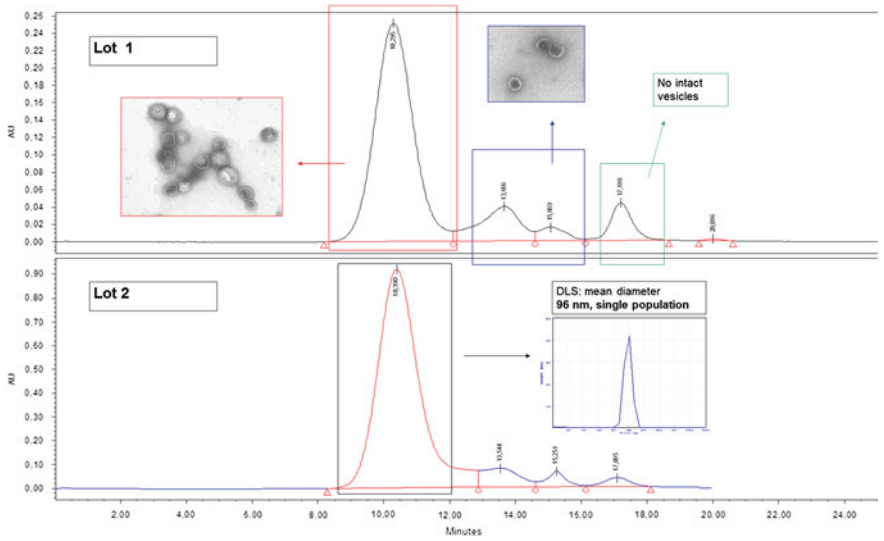


Fig. 6.10 Separation of OMVs on the basis of dimensions by SE-HPLC

Studies of OMVs subjected to different types of stress were used to evaluate whether the described analytical methods (DLS, TEM, SE-HPLC) were also able to monitor vesicles degradation.

Most of the stress conditions applied, i.e., thermal, pH, or physical stress, did not cause any degradation of the OMV particles demonstrating indeed the high degree of stability of this type of particles. Degradation of the vesicular structure was observed by the use of detergents. Zwittergents (3-(N,N-Dimethylpalmithylammonium propansulfonate) were found to be particularly effective in degrading the OMV membrane. The detergent was used as a 2 % solution in H_2O mixed with the OMV sample in a 1:1 ratio. The resulting solution was incubated at room temperature for at least 2 h before being analyzed by TEM and SE-HPLC analysis.

The analytical data on the OMV bulk material before and after the stress induced by Zwittergent are shown in the Figs. 6.11 and 6.12 and listed in Table 6.10. The Zwittergent caused a change in vesicle morphology as observed by TEM (Fig. 6.11). In Fig. 6.12, the main SE-HPLC peak (yellow frame) represents the integral OMV MenB NZ vesicles, while the additional peaks represent vesicle fragments. Addition of Zwittergent caused a major change in the chromatographic profile with complete absence of the peak representing the intact OMV particles. Batch DLS data presented in Table 6.10 show differences in the average OMV diameter before and after stress, but more significantly, a large difference in the polydispersity index caused by vesicle disruption.

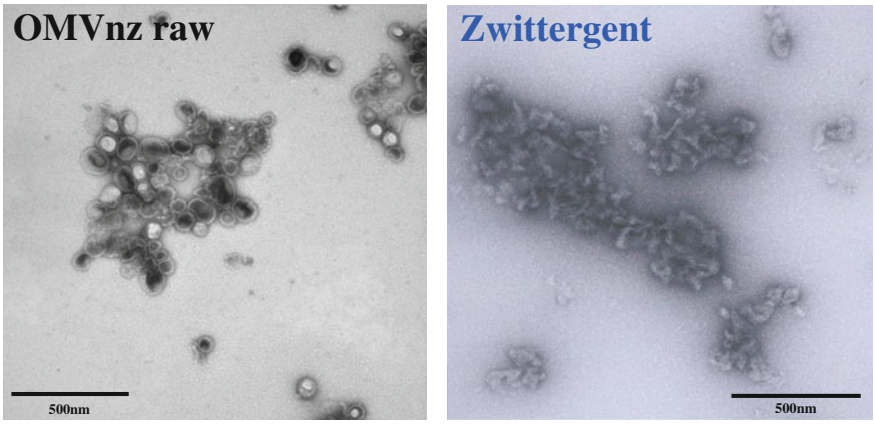


Fig. 6.11 TEM analysis of OMV before (*left*) and after (*right*) Zwittergent treatment. The bar at the *lower* part of the images represents 500 nm

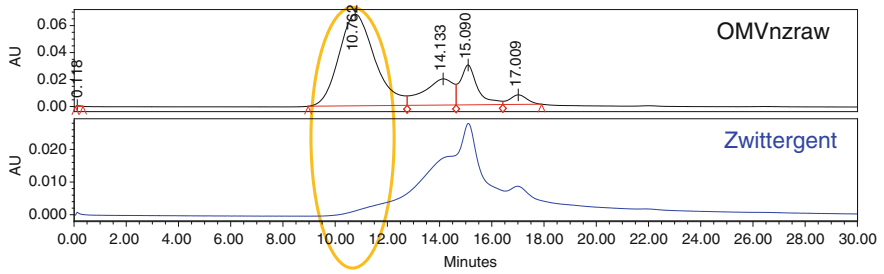


Fig. 6.12 SE-HPLC analysis of OMV before (*top*) and after (*bottom*) Zwittergent treatment. The *highlighted area* indicates the main vesicle peak, disappearing upon stress treatment

Table 6.10 DLS dimensional data on OMV before (top) and after (bottom) Zwittergent treatment

	Average diameter (nm)	PdI[a]
OMVnz raw	134	0.2
Zwittergent	114	1.1

The results represent the average of three repetitions
[a] Polydispersity index

6.3.2 OMV (MenB NZ) Protein Pattern and Content

Characterization of the OMV proteome has been accelerated by the availability of the *neisseria* genome sequence and of improved proteomic technologies. In addition to the most abundant outer membrane proteins, a high number of periplasmic and cytoplasmic proteins have been identified by 2D-PAGE and mass spectrometric

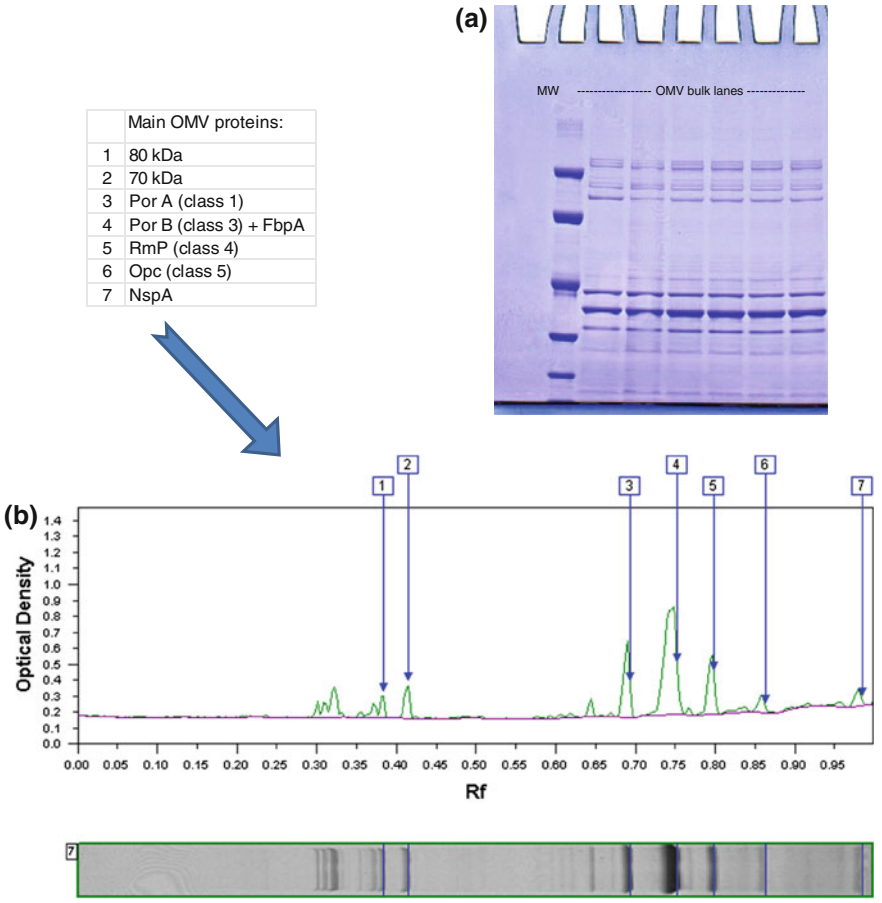


Fig. 6.13 Panel **a** SDS-PAGE separation of OMV bulk: *lane 1*, MW standard, other lanes, OMV bulk material; panel **b** single OMV lane electropherogram; other than relative ratio between target proteins (listed 1–7), their total percentage with respect to total proteins in the lane is evaluated [Figure adapted from Wassil et al. (2012) with permission]

analyses. In total, more than 100 proteins have been identified in DOC produced OMVs applying these technologies (Vipond et al. 2006; Ferrari et al. 2006).

The OMV protein pattern is quantitatively dominated by OMPs like PorA and PorB porins, RmpM (reduction modifiable protein), the OpcA invasin and iron-regulated proteins like FetA/FrpB, depending on iron-depleted growth conditions. Each of these proteins was suggested in the past to be possible vaccine candidates. Based on clinical study results, PorA is considered to be the immunodominant component of OMV following infant vaccination, although additional proteins may contribute to the immunogenic effect (Wassil et al. 2012).

SDS-PAGE with Coomassie Brilliant Blue staining and WB against specific OMPs have been the standard methods used to identify and determine the relative amount of the main protein antigens in the OMV bulk.

The SDS-PAGE release method monitors relative abundance of eight selected OMPs considered relevant both quantitatively and immunogenically in the presence of minimal quantities of several other less abundant proteins. Figure 6.13 shows a typical SDS-PAGE separation of the OMV proteins. Evaluation of the presence and relative quantities of the eight proteins identified as important for OMV immunogenicity with respect to the total OMV protein pattern is performed by densitometric evaluation of the gel lane electropherograms.

One of the specification criteria is based on the relative percentage of the single target proteins with respect to the total proteins based on band intensity in SDS-PAGE. A second specification criterion on total purity guarantees that the sum of the target proteins remains consistent with respect to nonspecified OMV proteins between different lots as ulterior assurance for standardized immunogenic response. The contribution of the nonspecified OMV proteins is distributed over a multitude of proteins that singularly are present in very small amounts and render accurate quantification challenging. These proteins are OMV derived and as such not impurities. Potentially they may be involved in the pathogenesis and thus providing immunity to meningococcal disease (Vipond et al. 2006).

RP-HPLC was applied on OMV bulk to develop a chromatographic method able to complement/confirm the SDS-PAGE separation method of the main OMV proteins. The chromatographic profile by UV detection showed the presence of several protein peaks (Fig. 6.14). To perform a preliminary identification of the observed peaks, total eluted volume plus three fractions of the RP-HPLC profile

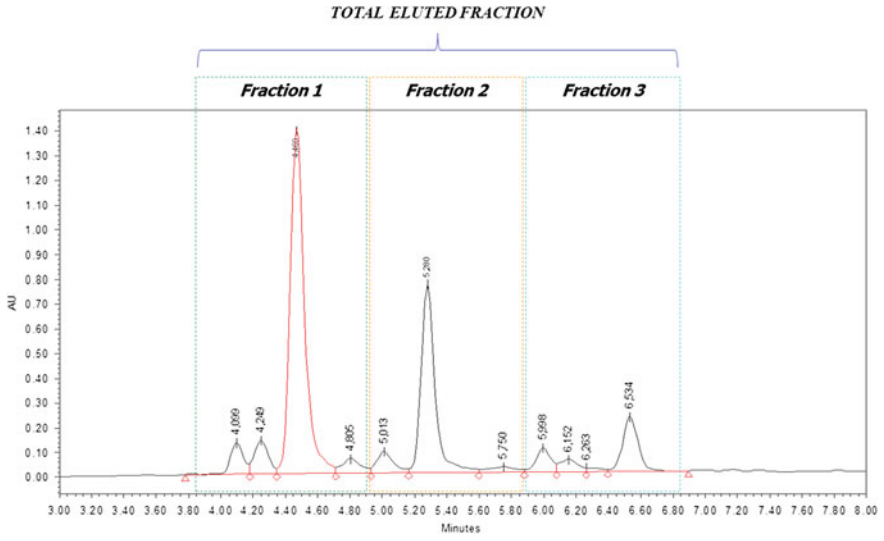


Fig. 6.14 RP-HPLC chromatographic profile of OMV and fractioning for peak identification

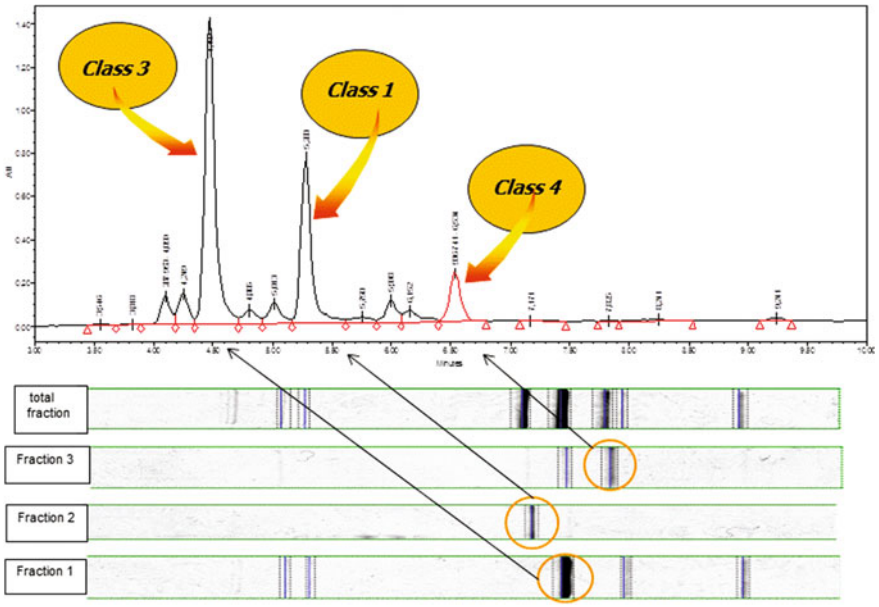


Fig. 6.15 The main protein peaks identified on the chromatogram (*top panel*) by comparison with SDS-PAGE of the corresponding fractions (*bottom panel*) are reported. The RP-HPLC method could provide advantages as potential release method. Identification of main OMPs in RP-HPLC separation (*top panel*) peak at 4.5 min assigned as *PorB class 3*; peak at 5.26 assigned as *PorA class 1*; and peak at 6.5 min *Rmp class 4*

were collected and analyzed by SDS-PAGE, reference SDS-PAGE characterization results were used for comparison (Fig. 6.15). Comparable profiles were obtained for OMV bulk directly loaded on SDS-PAGE and OMV total fraction, although differences in the relative protein concentration were observed, probably due to sample dilution with mobile phase during RP-HPLC elution.

In order to achieve a more comprehensive characterization of the OMV protein composition, mass spectrometric technologies are currently being developed. A large number of less abundant protein accounts for approximately 33 % of non-specified OMV proteins. These proteins are either difficult to detect or not detectable at all using SDS-PAGE or RP-HPLC. Consequently, more sensitive methods are required for their identification and quantification. Mass spectrometry is a powerful technology able to identify all proteins in very complex mixtures in a quantitative manner and represent an ideal tool for the analysis of the OMV proteome (Tani et al. 2014). The about 100 proteins of the OMV proteome and the limited dynamic range required to quantify most of these proteins allow for good coverage in a single LC-MS/MS run. Such a method will enable the assessment of lot-to-lot consistency on a proteome-wide level.

6.3.3 OMV (MenB NZ) LPS Quantification

LPS are structural constituents of OMV particles and cannot be avoided in detergent extracted material as mentioned earlier. However, endotoxicity of LPS in the final vaccine is reduced by two factors: first, the insertion of Lipid A into the vesicle membrane bilayer, and second, by adsorption of the OMV particles to Alum adjuvant in the final formulation.

Potential endotoxicity needs to be strictly monitored and therefore OMV bulk material is analyzed for LPS content in terms of total content via chromatographic determination and bioavailability via a chromogenic LAL test. In both cases, the values are expressed in relation to the total protein content.

The release method used for quantification of total LPS is based on the one developed by Lyngby et al. (2002). This analytical method determines the total quantity of LPS present in OMV independently of its bioavailability.

Quantification of LPS in the OMV is performed via determination of a fatty acid, 3-hydroxy-lauric acid (3-hydroxy-lauric, 3-OH-12:0), considered a marker molecule for the analysis because it is a specific part of the Lipid A component of *N. meningitidis* (Fig. 6.16). The method is based on precipitation of LPS from the OMV solution by addition of cold ethanol and NaCl, TFA (trifluoroacetic acid) hydrolysis for releasing fatty acids, their derivatization as phenacyl esters to allow quantification by RP-HPLC. A conversion factor taking into account fatty acid and LPS molecular weights, and the estimated 2:1 molar ratio between 3-OH-12:0 and LPS are then used to calculate the LPS content.

In order to ensure accuracy of the method, quantitative LPS precipitation from the OMV particles is critical. This cannot be evaluated by addition of the purified analyte 3-OH-12:0 since free fatty acids do not precipitate in the conditions used for LPS precipitation. Instead, purified commercial *Escherichia coli* LPS (a commercial rough strain from *E. coli* J5 Rc mutant) with a short chain of the lipopolysaccharide

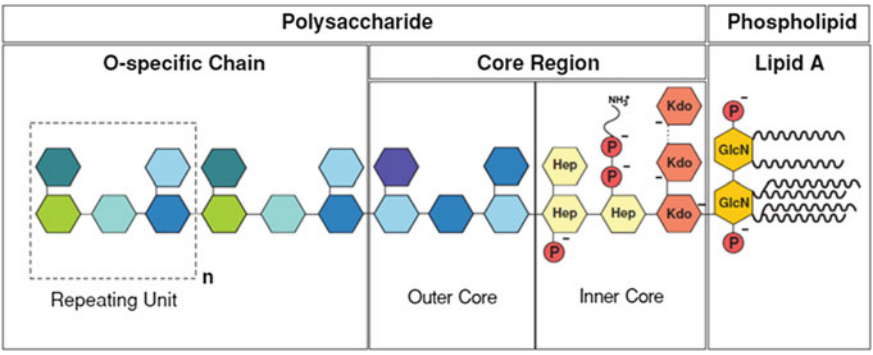


Fig. 6.16 *N. meningitidis* LPS structure: schematic representation of LPS, constituted by a polar oligosaccharide and nonpolar lipid portions; in addition by polar phosphates groups of *Lipid A* or other polar substituents that can decorate the core [Figure adapted from Alexander and Rietschel (2001) with permission]

(LPS), structurally related to *N. meningitidis* LPS was used successfully to demonstrate quantitative OMV precipitation.

OMV samples were spiked with purified *E. coli* LPS before precipitation; 3-OH-14:0 fatty acid, common to *N. meningitidis* and *E. coli* LPS was used for recovery evaluation as shown in Fig. 6.17.

Complete precipitation of LPS from OMV and accurate quantification was confirmed by GC-FID (Gas Chromatography with Flame Ionization Detector) in the precipitated OMV fraction by performing methanolysis on the precipitate followed by *n*-hexane extraction before GC analysis. Recoveries of 105 and 118 % with respect to the *E. coli* LPS spike were obtained.

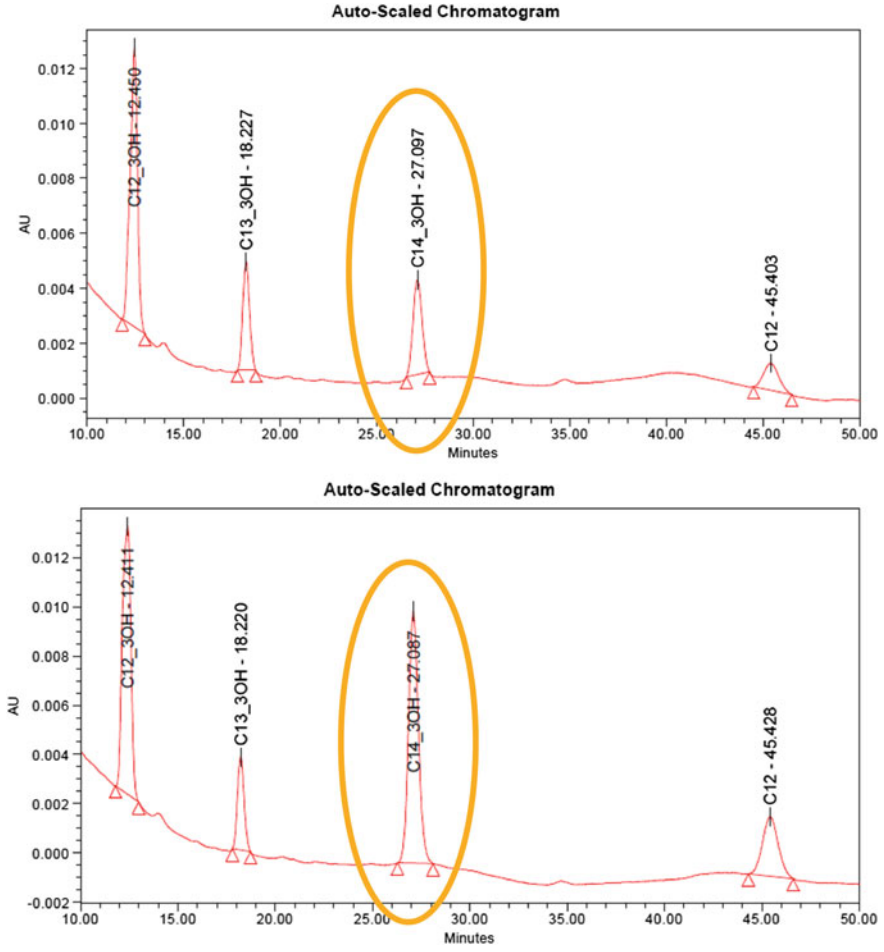


Fig. 6.17 Representative RP-HPLC chromatograms of OMV with (*bottom*) and without (*top*) *E. coli* LPS spike, respectively. The *peak* representing 3-OH-14:0 used for accuracy evaluation is indicated in the figure

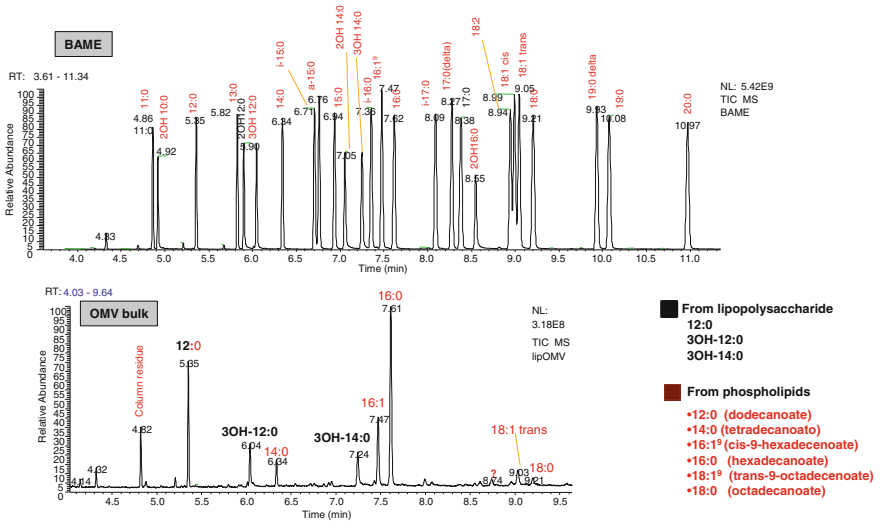


Fig. 6.18 Lipid pattern of OMV by GC-MS. GC profile of BAME standard (*top*) and GC profile of OMV bulk (*bottom*)

The GC analytical method was optimized to enable quantification of LPS in OMV without the need of the precipitation step. An additional advantage of this approach, besides the simplification of the analytical procedure, is the assessment of the complete lipid pattern of the vesicle.

Figure 6.18 shows a representative GC profile of OMV bulk with the optimized method without the precipitation step. Identification of the fatty acid peaks present in the sample was performed by comparison with a BAME (Bacterial acids methyl esters) standard analyzed under the same conditions and confirmed by MS/MS detection.

The analysis of fatty acids present in the OMV bulk shows the presence of short chains, mainly with 16, 14 and 12 carbons. The only unsaturated fatty acids are the 16:1 and 18:1. These findings are in agreement with literature data (Rahman et al. 2000) indicating as major phospholipids component of *N. meningitidis* the 16:0 acyl chain followed by the 16:1 and 14:0 fatty acids. The high abundance of 12:0 can be ascribed to it originating from both LPS and phospholipids.

6.3.4 OMV (MenB NZ) Other Components Characterization

Additional important component of the OMV particles are CPS (Capsular Poly-Saccharide) fragments deriving from the bacterial capsule. In fact, in the production process deoxycholate induces vesicle circularization as well as bacterial disruption and inactivation. Consequently, during vesicle formation part of the capsule is inserted in the OMV bilayer and not successively removed by purification.

MenB Capsular Polysaccharide is constituted by a polysialic chain α(2 → 8) N-acetyl neuraminic acid) of about 200 residues (about 80 kDa) in which the sialic acid (SA) is linked between position 2 and 8 (Fig. 6.19). This molecule retains structural homology to polysialylated components of human fetal and adult neural tissues resulting in a poor immunogen and precluding the development of an anti-MenB vaccine based on capsular polysaccharides. This is in contrast to MenC CPS, differing only in the SA linkage (between position 2 and 3), which is a highly efficacious vaccine (Khatami and Pollard 2010).

Although the theoretical risk that a MenB vaccine based on CPS might induce autoimmune disease in vaccinated persons was disqualified by several studies (Stein et al. 2006; Howitz et al. 2007), monitoring CPS presence in the OMV bulk intermediate remains important for product knowledge and process consistency reasons.

Presence of CPS in OMV bulk material was confirmed by DOT Blot using anti-CPS monoclonal antibodies and NMR. Quantification was performed by evaluation of CPS moieties by MEKC-UV (Micellar Electrokinetic Capillary Chromatography) and/or total SA quantification by HPAEC-PAD (High-Performance Anionic Exchange Chromatography—Pulsed Amperometric Detection) after chemical hydrolysis upon verification that SA contribution by sialylated LPS is negligible.

MEKC-UV can successfully separate small compounds as well as macromolecules, by use of a micelle–forming surfactant solution giving rise to separations into the fused silica capillary. The hydrophobic interactions between the analytes of interest and the micelles contribute to improve the separation selectivity, which is also based on the differential charge/radius ratio.

MenB CPS is partially degraded during the necessary lysis of the vesicles giving rise to a family of peaks with a specific electrophoretic mobility that can be baseline separated by MEKC and monitored by UV detection of the SA moieties. Although complex, such peak pattern allows a reliable CPS identification since a set of specific signals are associated to this relevant component. In Fig. 6.20, the Micellar CE profile (MEKC-UV detection) of an OMV sample after lysis (obtained by heating at 90 °C after SDS addition) is shown (OMV, middle). Comparing this

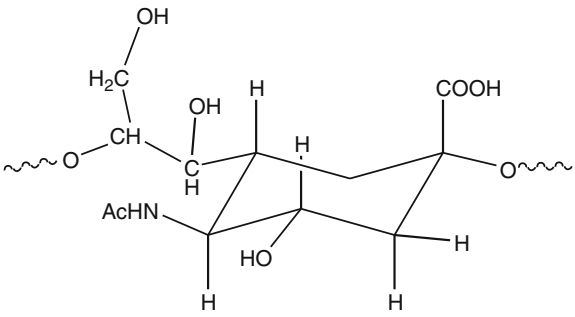


Fig. 6.19 Meningococcal group B CPS structure α(2 → 8) N-acetyl neuraminic acid)—a single moiety of SA is represented

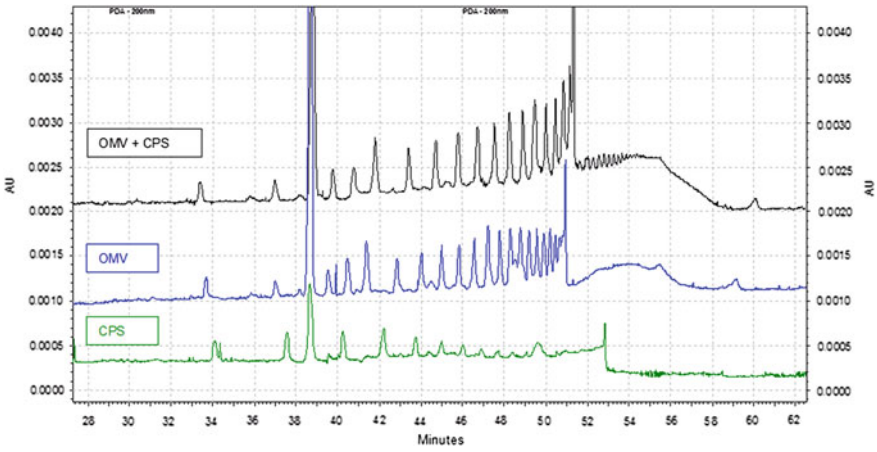


Fig. 6.20 MEKC-UV electropherograms of OMV (*middle trace*, *blue*), purified MenB CPS (*bottom trace*, *green*), and OMV + MenB CPS (*top trace*, *black*)

profile to that of purified CPS pretreated in the same way (CPS, bottom), it becomes clear that in the OMV sample, the polysaccharide component is present as a composite profile including several peaks corresponding to oligosaccharides of different length as demonstrated by an OMV sample spiked with CPS before lysis (OMV+ CPS, top).

Due to matrix complexity, quantitative CPS evaluation in OMV required building the standard curve directly on the sample by spiking of different standard amounts (standard additions method). In this case, purified commercial colominic acid (CA), presenting the same structure as MenB CPS, was used as quantification standard.

Figure 6.21 shows in the upper panel superimposed electropherograms of an OMV-bulk sample spiked with different CA standard amounts. Traces are normalized on the internal standard signal present in each run. The lower panel presents the related standard curve, built by plotting on the *x* axis of the graph the values of mg/ml of CA standard added to the sample, and on the *y*-axis, the value of the sum of the areas of all the peaks belonging to the CPS distribution. Using this calibration curve, the CPS content of the original OMV sample can be calculated extrapolating the concentration value at zero addition.

The CPS content determined using MEKC-UV and expressed as CPS/OMV total proteins ratio for more than 20 OMV lots resulted always lower than 5 %.

Alternatively, MenB CPS can be determined applying different strategy: acidic hydrolysis of CPS to SA units and quantification via HPAEC-PAD chromatography, using conditions suitable for monosaccharide separation.

Figure 6.22 presents the HPAC-PAD elution pattern of an OMV-bulk sample after hydrolysis. Apart from the SA peak, other monosaccharides released from chemical hydrolysis of the sample and present in the chromatogram were identified by use of the appropriate purified standards run under the same chromatographic

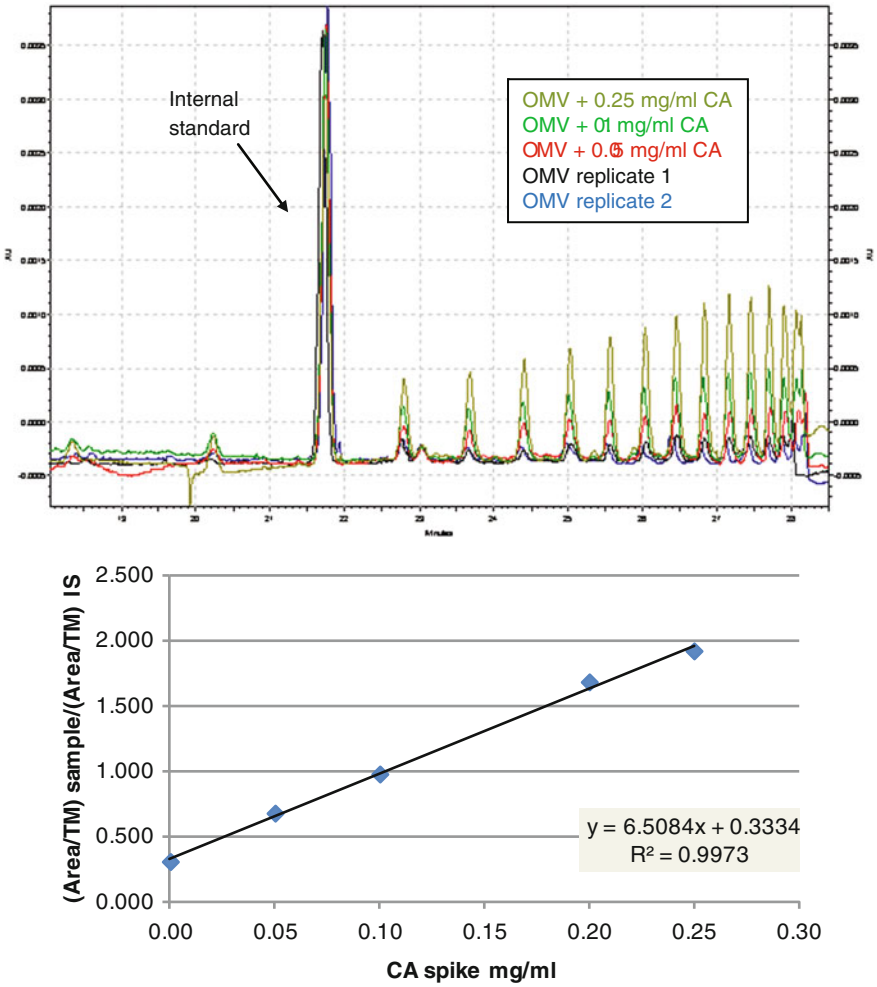


Fig. 6.21 *Upper panel* superimposed electropherograms of an OMV bulk sample spiked with different CA standard amounts; *lower panel* example of resulting standard curve

conditions: KDO, from the LPS inner core, glucose and fructose mainly as components of the sucrose added as stabilizer, and glucose from OMV glycosylations. Fucose was added as internal standard.

With the HPAEC-PAD quantification method, the CPS/protein ratio was determined to be in the range of 2–6 %, in line with the MECK-UV results.

Since an additional source of SA can be sialylated LPS in OMVs, the SA released by a chemical hydrolysis approach accounts for both CPS and LPS. Hydrolysis by enzymatic means, utilizing different neuraminidases, can alternatively break any SA linkage α(2 → 3, 6, 8, 9), or selectively only the α(2 → 3, 6),

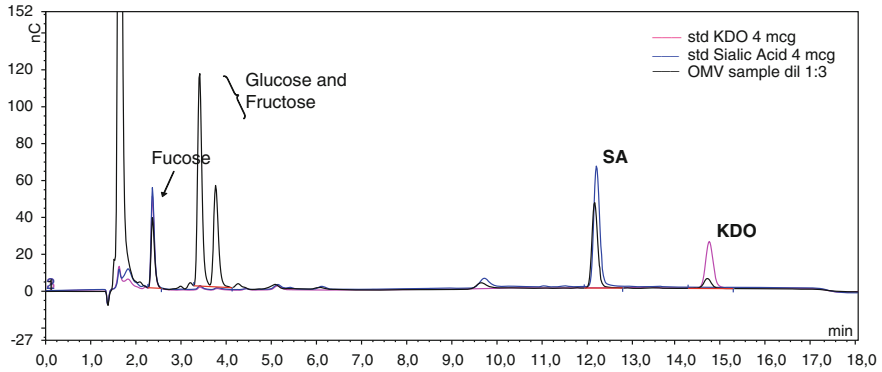


Fig. 6.22 HPAC-PAD elution profile of OMV after chemical hydrolysis

related to LPS, allowing to determine the CPS contribution of SA. The contribution to SA by LPS evaluated by the enzymatic approach was found to be negligible with respect to that deriving from CPS.

Potentially other components of the bacterium could be included in OMVs during the vesicle budding process. Further characterization was mainly focused on those molecules that could induce endotoxin-like actions, i.e., stimulating a complex intracellular signaling pathway that leads to the production of inflammatory cytokines, such as IL-6, IL-1, and TNF (McDonald et al. 2005).

One set of studies was aimed to verify the presence of the bacterial peptidoglycan (PGN) in OMV of *N. meningitidis* group B bulk samples. PGN is composed of carbohydrate chains of β-(1 → 4)-linked, *N*-acetylglucosamine and *N*-acetylmuramic acid, cross-linked by short peptide chains (Vollmer et al. 2008), as schematically reported in Fig. 6.23.

PGN presence was evaluated through the identification by GC of a specific and univocal component of PGN, monosaccharide N-acetyl-muramic acid, whose structure is presented in Fig. 6.23.

Monosaccharides from OMV bulk samples were analyzed by GC as acetylated O-methyl glycosides. Because of the presence of 3 % sucrose in the matrix, samples were ultrafiltered to reduce the sucrose content, then derivatized with methanolic HCl and a successive extraction with *n*-hexane allowed to remove the lipid components as FAME (Fatty Acid Methyl Esters).

The methanolic polar phase, containing the OMV monosaccharides as acetylated O-methyl-glycosides, was analyzed by GC with mass spectrometric detection equipped with a positive electron ionization mode source to allow univocal components identification through retention time comparison with standards and mass fragmentation pattern of the monosaccharides.

Figure 6.24 reports a representative GC-MS profile for OMV bulk in relation to its monosaccharide content. The acetylated O-methyl glycoside profile obtained confirmed the presence of monosaccharides belonging to the lipooligosaccharide

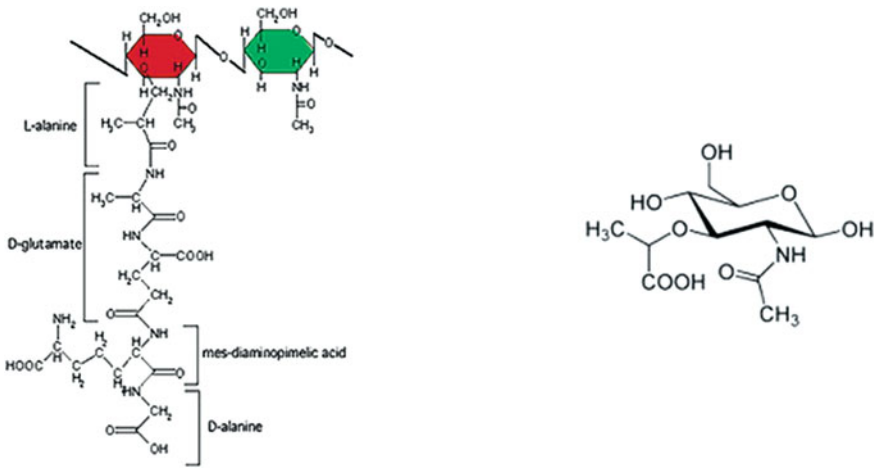


Fig. 6.23 Schematic representation of bacterial peptidoglycan, *left side*, and structure of NAM N-acetyl-muramic acid (2-N-acetyl-3-O-lactyl-glucosamine), *right side*

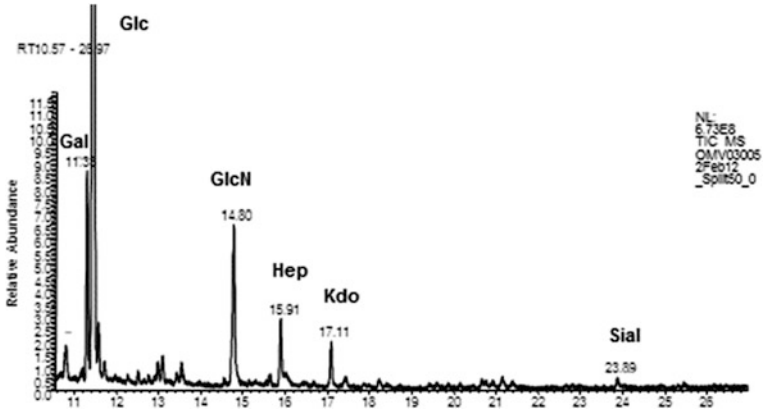


Fig. 6.24 GC-MS chromatogram (enlarged) of acetylated O-methyl glycosides of OMV bulk. Glucose comes from remaining traces of sucrose in the sample

structure: Gal, GlcN, Hep, Kdo, sialic acid. The latter can either derive from lipooligosaccharide or capsular polysaccharide (Pavliak et al. 1993) since it is a constituent of both structures. The predominance of glucose originates from sucrose (contained in 3 % as stabilizer) that was not entirely removed by ultrafiltration.

No traces of muramic acid (Mur) were detected in the saccharidic pattern of OMV. To confirm these results, Fig. 6.25 presents a comparison of the standard Mur elution (19.5 min, black trace) with the Mur spiked OMV sample (red trace), and the OMV sample without spike (blue trace). In the OMV sample without spike, no peaks corresponding to Mur were identified.

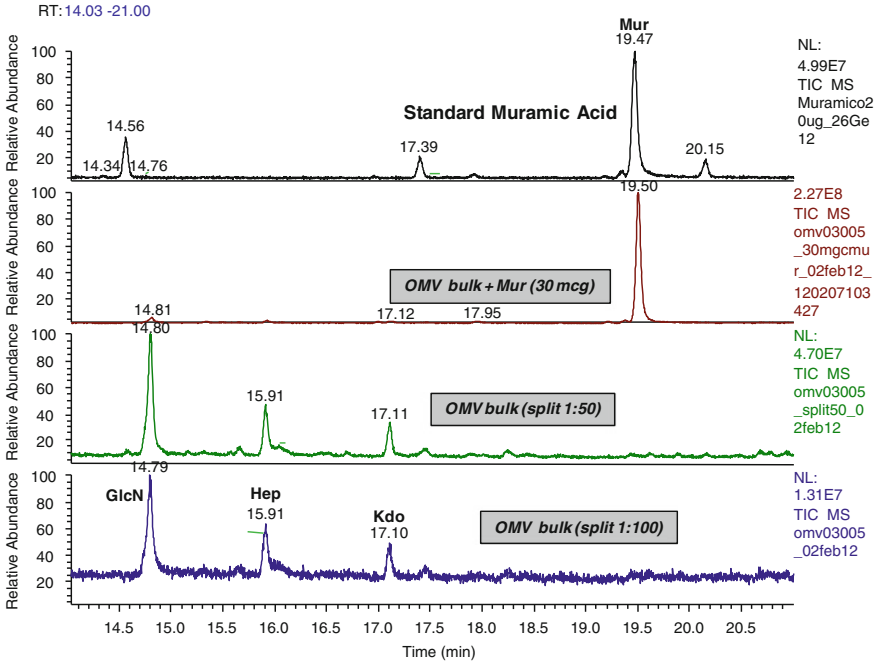


Fig. 6.25 Overlay of GC-MS chromatograms from OMV bulk w/o Mur spike, and Mur standard. The OMV sample without spike was analyzed with two different split arrangements

To increase sensitivity, a split of 1:50 was also used for OMV sample injection (green trace). The split allows a part of the sample injected by the syringe to be vaporized and escape through the split vent. A split ratio 1:100 (as normally used for the presented analyses) indicates that 100 parts are injected and 1 part goes to the column. A twofold quantity of sample is analyzed by split 1:50. The absence of Mur was confirmed also in this case.

The analysis was repeated on four lots. In all cases, no N-acetyl muramic acid was detected by the comparison of OMV sample w/o spike of the target Mur monosaccharide.

6.3.5 Overall Conclusions

The concentrated MenB OMV vaccine/vaccine intermediate is a stable colloidal suspension that consists of vesicles preserving the subcapsular cell surface composition of *N. meningitidis* serogroup B.

Due to the complex structure and presence of several antigenic components, the OMV particles present several analytical challenges. Here, we have described most of the classical and novel analytical methods required to characterize the OMV

particles at a structural and molecular level. Chemical composition and quantity of the single constituents are necessary but not sufficient for a complete characterization of OMV as a vaccine antigen. A comprehensive dimensional and morphologic characterization of the OMV particles can be obtained only by applying a number of analytical methods that target different properties of sizing and appearance. DLS, EM, and SE-HPLC represent complementary methodologies to detect vesicle degradation in forced degradation studies.

The OMV protein pattern is quantitatively dominated by OMPs like PorA and PorB porins. PorA is considered the immunodominant constituent of OMV in infants, but additional proteins may contribute to the immunogenic effect. The identity and relative amount of the main protein antigens in the OMV bulk are classically determined by the analysis of specific OMPs by SDS-PAGE with Coomassie Brilliant Blue staining and WB. In order to achieve a more comprehensive characterization of the OMV protein pattern mass spectrometric technologies are currently being developed. Mass spectrometry has been proven to be capable of simultaneously identifying and quantifying proteins out of complex mixtures and is ideally suitable for the OMV proteome.

LPS presence in OMV is monitored at bulk release both as total quantity and as bioavailable LPS. In addition to the physicochemical release test method by RP-HPLC specific for the MenB LPS quantification, an orthogonal GC-FID method has been developed to confirm LPS quantification results and to obtain a more comprehensive view of the OMV lipid pattern.

Presence of additional potential immunogenic OMV components was evaluated in addition to OMPs and LPS. The focus was on components that could have an impact on the safety and potency of the vaccine. The absence of significant quantities of PGN was verified. In addition, orthogonal methods for the monitoring of CPS were demonstrated to give consistent and reliable results.

6.4 Cholera Vaccine: Cholera Toxin B Subunit Derived Vaccine

6.4.1 Introduction

V. cholerae is a Gram-negative bacterium and the causative agent of cholera. Cholera is one of the dreaded epidemic and pandemic diseases. The disease has the unusual ability to spread rapidly to large numbers of people, to spread internationally and to kill a high proportion of those affected (WHO Cholera: global surveillance summary 2008; Bishop and Camilli 2011). Cholera is a rapidly dehydrating, watery diarrheal disease caused by intestinal infection with the bacterium *V. cholerae*, mainly of serogroup O1 that produces cholera toxins (CT). The division of *V. cholera* into O serogroups is based on its major surface antigen, the heat stable endotoxin. The endotoxin is a LPS, where the lipid portion of the

molecule is embedded in the cell wall and polysaccharide extends out from the surface of bacteria (Bishop and Camilli 2011). The classical biotype predominated up until the current pandemic, when the El Tor biotype surfaced and has since entirely replaced the classical biotype. Both the classical and O1 El Tor biotypes consist of two O1 serotypes: Ogawa and Inaba. A new serogroup, O139 was reported to be causing significant cholera-like disease in India and Bangladesh in 1992 (Ramamurthy et al. 1993; Cholera Working Group ICDDR, Bangladesh 1993; WHO 1991; 2000; Sack et al. 2004). Serogroup O139 appears to have arisen from O1 El Tor by the acquisition of a new LPS and capsule-encoding locus (Waldor and Mekalanos 1994). In fact, outside of the LPS locus, O139 shares the majority of traits with O1 El Tor. Although both serogroups O1 and O 139 can elicit serum antitoxin responses, infection with one serogroup has not been shown offer cross protection against the other (Albert et al. 1994). By contrast, the O1 El Tor and classical biotypes, despite having identical O-antigen loci, exhibit many genotypic and phenotypic differences.

V. cholerae survives passage through the stomach and colonizes the epithelial surface of the small intestine. *V. cholerae* uses adhesion factors, some of which may remain to be elucidated, that include: O1 LPS (Chitnis et al. 1982); GlcNAc-binding protein (GbpA) (Kirn et al. 2005); a protein (TcpF) secreted by the toxin coregulated pilus (TCP) biogenesis apparatus (Kirn and Taylor 2005); outer membrane protein OmpU (Sperandio et al. 1995); and CT, although this has only been implicated in an adult rabbit model (Pierce et al. 1985). TCP facilitates inter bacterial interactions that are important for colonization (Taylor et al. 1987; Herrington et al. 1988; Asaduzzaman et al. 2004). An effective cholera vaccine could prevent colonization by inducing the production of antibodies that directly neutralize the function of key colonization factors and/or facilitate phagocytosis and killing through bacterial opsonization (Bishop and Camilli 2011).

6.4.2 Cholera Vaccines

The following oral cholera vaccines are licensed and available without and with rCTB:

1. Killed Whole Cell (WC) of *V. cholerae* O1 (classical and El Tor, Inaba and Ogawa) plus recombinant CT B (rCTB*) subunit vaccines—Dukoral™.
2. Killed WC (Modified WC-only) of *V. cholerae* (serogroups O1 classical and E1-Tor; possibly O139) and no clinical evaluation to date. This vaccine is formulated without rCTB vaccines—Shanchol™ or mORCVAX.
3. Killed Whole Cell-only of *V. cholerae* (serogroups O1 classical and E1-Tor; possibly O139) and no clinical evaluation to date—ORC-Vax™.

In this chapter section, we focus on CT derived B subunit protein (CTB) containing cholera vaccines and the vaccine analytical characterization and testing strategy for drug substance (bulk) and drug product (final product) release, stability,

and rationale for inclusion of recombinant rCTB based on guidelines for the production and control of inactivated oral cholera vaccines (WHO requirements as adopted in 2001; Annex 3, Guidelines for the production and control of inactivated oral cholera vaccines, WHO Technical Report, Series No. 924, 2004).

More details on licensed and in-use whole-cell inactivated and live-attenuated cholera vaccines can be found in Chap. 5 in this book.

6.4.3 *Dukoral™*

Dukoral was developed in Sweden and first licensed in 1991 and is currently produced by Crucell. It is licensed in >60 countries, primarily as a vaccine for travelers to cholera-endemic areas. However, it has also been used in crisis situations in Indonesia, Sudan, and Uganda, and in a demonstration project in an endemic area of Mozambique. Dukoral is a monovalent vaccine based on formalin and heat-killed WCs of *V. cholerae* O1 (classical and El Tor, Inaba and Ogawa) plus rCTB subunit (see Package Insert and also Chap. 5 of this book). The B subunit of CT was originally produced chemically (WC-BS) but is now produced by recombinant technology (WC-rBS). B subunit and recombinant B subunit are identical in terms of immune response (Jertborn et al. 1992; Sanchez and Holmgren 1989). To protect the CT B subunit from being destroyed by gastric acid, the vaccine should be given with a bicarbonate buffer (Clemens et al. 1986). The vaccine is provided in 3-ml vials together with the bicarbonate buffer (effervescent granules in sachets). Vaccine and buffer are mixed in 150 ml of water for persons aged >5 years and in 75 ml of water for children aged 2–5 years (Crucell Product Package Insert and Chap. 5).

6.4.4 *Rational for Inclusion of Cholera Toxin B Subunit (CTB) in the Vaccine*

Cholera toxin is an oligomeric protein complex secreted by the bacterium *V. cholerae* and is the pathogenic agent responsible for the symptoms of cholera. The holotoxin, like many other bacterial toxins, is built up from two subunits A and B. The A-subunit, itself built up by two peptides A1 and A2, exerts the toxic enzymatic activity of the toxin. The B-subunit, in the form of a homopentamer, binds the toxin heterohexamer to GM1 ganglioside receptors on mammalian cell surfaces and facilitates entrance of the A subunit into the cell but has no toxic effect in itself. The A subunit bears the ADP-ribosyl-transferase activity, which deregulates the G protein causing activation of adenylate cyclase. The B subunit alone is considered nontoxic. In vitro, CT B subunit (CTB) is an efficient mucosal adjuvant and carrier molecule for the generation of mucosal antibody responses and/or induction of

systemic T-cell tolerance to linked antigens. Due to the ubiquitous occurrence of the GM1 ganglioside receptor on eukaryotic cell membranes, CTB can be used in a wide variety of model systems. CTB is also used for tract tracing in neurological research, taking advantage of its high affinity to GM1 gangliosides and retrograde transport (Tayot et al. 1981; Spangler 1992; Merritt et al. 1994, 1998).

The vaccine contains whole-cell bacteria to create an antibacterial response and the B subunit of CT to create an antitoxin response. A majority of the antibodies against CT, obtained either after natural disease or immunization, is directed against the B-subunit. Also, because most part of the A-subunit is embedded within the ring-formed homopentameric structure of the B-subunit (see Fig. 6.1), antibodies against the A subunit, even if they were induced, would have little effect on the neutralization of the toxin. Using the B-subunit alone instead of the whole CT molecule in the oral cholera vaccine will thus not noticeably reduce the neutralizing immune response (EMA 2005). The B subunit exists as a homopentamer surrounding the toxic A subunit (CTA) (Merritt et al. 1994, 1998; Dakterzada et al. 2012). A response to the B subunit will also neutralize the toxic effect of the CTA (Merritt et al. 1998). This is the rationale for inclusion of CTB in the vaccine. The current vaccine (Dukoral™) formulation contains recombinant XT B subunit (rCTB) (Fig. 6.26).

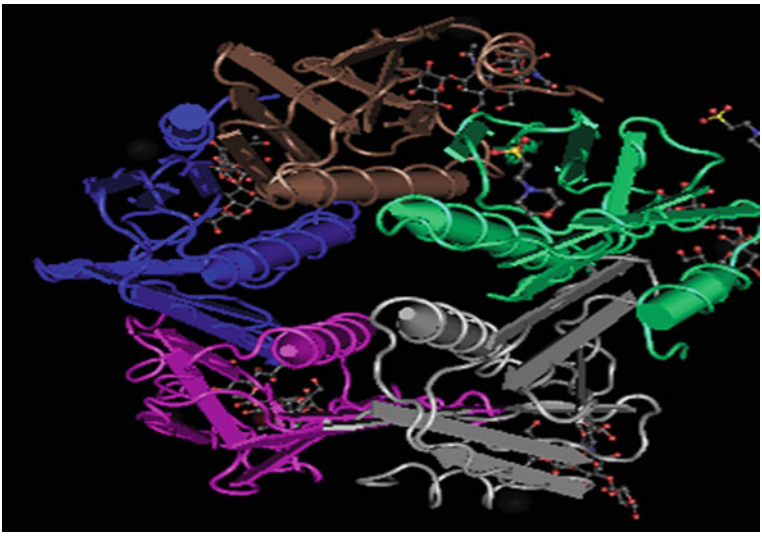


Fig. 6.26 X-ray diffraction structure of Cholera Toxin B-pentamer complexed with GM1 Pentasaccharide ganglioside receptors on mammalian cell surfaces and facilitates entrance of the A subunit into the cell (The 1.25 Å resolution). Figure adopted from reference Merritt et al. (1998). Printed with permission of NCBI NIH http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid=8325

6.4.5 Cholera Vaccine Product Development

The vaccine is formulated as an oral suspension. The antigens are aseptically mixed with PBS and filled in 3.0 ml doses (EMEA 2005, Crucell Product Package Insert). This dose of the vaccine was chosen based on knowledge about intestinal immunity, indicating that the oral route is superior to the parenteral for stimulating an immune response in the gut (Jerborn et al. 1993). The only additive PBS was chosen to control the pH and salt concentration, in order to improve the stability of the antigens. Regardless of the fact that the vaccine is intended for oral administration and it is formulated as a sterile product (see Table 6.11 for details).

A bicarbonate buffer is included in the product and is taken with the vaccine in order to neutralize the stomach acid that otherwise destroys rCTB. The phosphate-buffered saline (PBS) consist/ml of Sodium dihydrogen phosphate monohydrate 0.576 mg Disodium phosphate dihydrate 3.13 mg, Sodium chloride 8.5 mg and water for injections add 1 ml (see Product Package Insert, Crucell) (Table 6.12).

Each ingredient is tested and released as per the standard methods described in EP and these analytical methods can be found in the Pharmacopeia.

6.4.6 Drug Substances (Final Bulks)

The whole-cell bulks are produced using standard manufacturing procedures (cGMP). A routine in-process and release test for this has been introduced. The bacterial bulks are produced from established seed-lot systems that are controlled by standard microbiological and biochemical methods generally used in vaccine production WHO 1992, Annex 1 (WHO Technical Report, Series No. 822); WHO 2004 Annex 3 (WHO Technical Report, Series No. 924).

Table 6.11 The complete composition of the WC of *V. cholerae* O1 (classical and El Tor, Inaba and Ogawa) plus recombinant cholera toxin B (rCTB) subunit vaccines is given

Ingredient	Reference	Amount	Function
Recombinant CTB		1 mg	Active ingredient
Vibrio cholerae O1 Inaba classical biotype, heat inactivated	Ph. Eur.	25×10^9 bacteria	Active ingredient
Vibrio cholerae O1Inaba El Tor biotype, formalin inactivated		25×10^9 bacteria	Active ingredient
Vibrio cholera O1Ogawa classical biotype heat inactivated		25×10^9 bacteria	Active ingredient
Vibrio cholerae O1Ogawa classical biotype, formalin inactivated		25×10^9 bacteria	Active ingredient
Phosphate-buffered saline pH 7.2–7.4		Ad 3 ml	Buffer

Table 6.12 The buffer sachet contains the following ingredients as specified in European Pharmacopeia

Ingredient	Reference	Amount	Function
Sodium hydrogen	Ph. Eur.	3. 6 g	Effervescentagent
Carbonate			
Citric acid anhydrous	Ph. Eur.	1.45 gm	Effervescent agent
Raspberry flavor	Monograph	70 mg	Aroma
Saccharin sodium	Ph. Eur.	30 mg	Sweeting agent
Sodium carbonate	Ph. Eur.	400 mg	Effervescent agent
Sodium citrate	Ph. Eur.	6 mg	Effervescent agent
Water, purified	Ph. Eur.	Disappears during manufacture	Granulating liquid

The active substances (bulk vaccine) are

1. rCTB subunit and
2. Four whole-cell bulks;

2a. *V. cholerae* O1 Inaba, classical biotype, heat inactivated,
2b. *V. cholerae* O1 Inaba, El Tor biotype, formalin inactivated
2c. *V. cholerae* O1 Ogawa, classical biotype heat inactivated and
2d. *V. cholerae* O1 Ogawa, classical biotype, formalin inactivated.

6.4.7 Vaccine Product and Analytical Development

6.4.7.1 RCTB Bulk (Drug Substance)

Initially during the development of vaccine production native CTB was used (the initial clinical studies) (EMEA 2005; Clemens et al. 1986; Peltola et al. 1991), and the current vaccine contains a recombinant CTB (rCTB) (EMEA 2005; Scarpella et al. 1995). Both molecules have been extensively characterized and shown to be comparable and identical, except for six short peptide native CTB extensions at the N-terminal in rCTB (EMEA 2005; WHO 2004). The rCTB is a protein of 102 amino acids containing one disulphide bridge (cys 9-cys 86). It is produced in *V. cholerae* strain 213 of serotype Inaba, biotype classical with a deleted CTA gene.

6.4.7.2 Characterization of rDNA-Derived B-Subunit (RCTB)

Rigorous characterization of the rDNA-derived B-subunit product should be undertaken using a variety of analytical techniques exploiting several different properties of the molecule, including size, charge, and amino acid composition.

Techniques suitable for such purposes include SDS-PAGE, size exclusion, and reverse-phase chromatography. Sufficient sequence information should be obtained by direct sequencing and by peptide mapping, or another appropriate molecular techniques. For example, mass spectrometry, in comparison with natural CTB. The identity of the product should be confirmed by at least partial N-terminal and C-terminal amino acid sequencing. Several lots of the product should be as fully characterized as possible (Annex 3, WHO 2004).

6.4.7.3 Biophysical Characterization of RCTB

Mass Spectrometry Analysis

Five or six molecular species with different molecular mass corresponding to different N-termini were found in MS analysis (EMEA 2005). The findings are consistent with what was found in N-terminal analysis where six different N-termini were detected, all of them extensions of the native CTB up to seven amino acids. No truncated forms compared to native CTB were found. The results are due to the construct of the vector and expression cassette and the variants found were the expected, based on the construct and forms in between (EMEA 2005).

RP-HPLC Analysis

After long time storage of the rCTB under refrigerated conditions and shorter under RT, there is a tendency of a shoulder of the peak found in the RP-HPLC which is explained to be related to the presence of low proteolytic enzyme activity, probably due to a type I signal peptidase. In no case has a sequence shorter than the native CTB been seen and the protease activity has been shown to decrease in later manufacturing batches. In view of the route of administration, the risk associated with the peptidase activity in the formulation is negligible when administered to humans and of no significance to the safety of the product (EMEA 2005).

MS have been used to determine the molecular weight and functional tests (GM1 binding, binding to monoclonal antibodies) have been performed. The DNA sequence has been confirmed and it is deemed unlikely that a protein emanating from the correct DNA sequence, with the molecular weight expected from the N- and C-terminal analyses and reacting comparably with native CTB in functional assays would differ from the expected structure (EMEA 2005).

Analytical methods and release specifications for rCTB bulk

rCTB bulk analytical methods and release specifications are shown in Table 6.13.

rCTB bulk stability indicating methods and release specifications are shown in Table 6.14.

Table 6.13 Examples of rCTB (as a component of recombinant cholera toxin B subunit) bulk release specifications

Critical quality attributes	Analytical method	Release specification	Rationale
Physical appearance	Visual inspection	Clear, colorless to weakly yellow solution. Some particles may occur	This test evaluates physical appearance of the bulk solution and should be free of aggregates and extraneous particles
pH	Potentiometry	7.0–7.6	The test measures pH of the whole-cell bulk suspension
Identification	Ouchterlony immunoelectrophoresis	Immunological identity with rCTB and CTB	The test measures immunological identity with rCTB and CTB by specific B subunit antibodies
Sterility	Membrane filtration (Ph. Eur.)	Sterile	The test measures sterility of the rCTB drug substance, required before vaccine formulation
Purity	RP-HPLC	<10 % unrelated proteins	The test evaluates the total % of unrelated proteins in the rCTB bulk by reverse-phase chromatography
Purity	SDS-PAGE	Not more than 2 bands visible; one major at 12 kD and one minor if present at 23 kD	The test measures by electrophoresis the presence of antigen bands. One major band representing rCTB and a minor band representing high molecular weight. It also evaluates purity of the bulk
Purity	SE-HPLC	Area of pentamer peak >90 % of integrated area	Size exclusion chromatography determines area of B subunit pentamer peak of rCTB and % of integrated area
Antigen concentration	Mancini	>1 mg rCTB/ml	The test measures antigen concentration as measured by single radial immunodiffusion
Protein content	Kjeldahl	>1 mg protein/ml	The test measures protein nitrogen and

(continued)

Table 6.13 (continued)

Critical quality attributes	Analytical method	Release specification	Rationale
			estimates protein content in the bulk
Antigenic purity	Antigen content/protein content	NLT 0.8 mg rCTB/mg protein	The test measures absorbance at 280 and 310 nm to determine rCTB purity. It evaluates the ratio of rCTB and protein content in the bulk

Table 6.14 rCTB stability indicating methods and release specifications

Critical quality attributes	Analytical method	Release specification
Physical appearance	Visual inspection	Clear, colorless to weakly yellow solution. Some particles may occur
pH	Potentiometry	7.0–7.6
Sterility	Membrane filtration (Ph. Eur.)	Sterile
Purity	RP-HPLC	<10 % unrelated proteins
Purity	SDS-PAGE	Not more than 2 bands visible; one major at 12 kD and one minor if present at 23 kD
Purity	SE-HPLC	Area of pentamer peak >90 % of integrated area
Antigen concentration	Mancini	>1 mg rCTB/ml
Protein nitrogen	Kjeldahl	>1 mg protein/ml
Antigen content/protein content	Absorbance at 280 and 310 nm by spectrophotometry	NLT 0.8 mg rCTB/mg protein

rCTB bulks are stored for a period of 36 months at 2–8 °C and it is substantiated by stability data. A shoulder is seen in the main peak in RP-HPLC chromatograms of samples stored for 6 months at 25 °C. Upon even further storage, this shoulder may even turn into a distinct peak and could also be detected in samples stored at 5 °C. This phenomenon was not observed in samples stored at −70 °C. It was shown that this was the result of cleavage of the longer N-terminal extra amino acids present in rCTB into shorter extensions, due to the presence of minute amounts of the signal peptidase I, responsible for cleavage of the signal peptide at the N-terminal of the rCTB molecule. However, no cleavage product shorter than native CTB has been found (EMEA 2005).

Whole-cell monovalent bacterial bulk (Drug Substance) analytical methods, release and stability specifications are shown in Table 6.15.

Table 6.15 Stability indicating release specifications for monovalent bacterial bulks

Critical quality attributes	Analytical method	Release specification
Physical appearance	Visual inspection	Beige opalescent suspension
Sterility	Ph. Eur. direct inoculation	Sterile
Homogeneity	Visual	Homogeneous suspension. No visible aggregation
Innocuity	Mouse weight-gain assay	No decrease in weight, no signs of morbidity
pH	Potentiometry	6.7–7.6
O1-LPS content	Inhibition ELISA	Tested and reported

Drug Substance Stability Indicating Methods (Whole-Cell bulks)

Whole-cell monovalent bacterial bulk (Drug Substance) stability indicating methods, and release specifications are shown in Table 6.16.

Whole-cell bulks (heat and formalin inactivated) are stored for a period of 3 years under refrigerated conditions and it is substantiated by stability data. The testing meets the EP and the WHO requirements (WHO 1992, Annex 1 (WHO Technical Report, Series No. 822); WHO 2004 Annex 3 (WHO Technical Report, Series No. 924). The other ingredient of the preparation is a PBS which constituents and testing meet the EP requirements (Ph. Eur.).

Table 6.16 Release and shelf life specifications for final bulk lot

Test attribute	Test method	Specification
Physical appearance	Visual control	Beige opalescent suspension
PH	Ph. Eur.	6.5–7.4
Homogeneity	Visual control	Homogeneous suspension
Purity	Gram staining	
Antigen concentration		Only gram-negative rods
O1-LPS	Inhibition ELISA	No visible aggregation
rCTB	Mancini	≥750 ELU/dose
Residual formaldehyde	Ph. Eur.	0.8–1.2 mg/dose
Sterility	Direct inoculation	<6.7 mM
		No microbial growth
Identity	Slide agglutination	Agglutination with Inaba and Ogawa specific antibodies
		Identity with B subunit (rCTB/CTB)
	Ouchterlony	

Drug Product Formulation

The vaccine is formulated with the monovalent cholera bulks (see Table 6.11). As the bacteria are inactivated, live count can for this reason not be performed. The amount added is instead added based on the bacterial content prior to inactivation. The PBS buffer, the monovalent bulks and the rCTB bulk is aseptically mixed and stirred to homogeneity. Each vial is sealed with a rubber stopper and a screw cap with a safety ring (EMEA 2005).

The vaccine production process has been appropriately validated and is substantiated by batch analysis data, which conform to the preset acceptance criteria for including the final lot and final bulk specifications. Homogeneity of the bulk was maintained throughout the filling process, as demonstrated by measurements of the optical density at 600 nm of samples withdrawn during and after filling. Environmental monitoring and media fills also showed the aseptic nature of the process. Thus, the production process for the vaccine is shown to be suitable and to consistently yield a product of the desired quality (EMEA 2005).

Final bulk lot of vaccine release analytical methods and their specifications are shown in Table 6.16.

Final bulk lot of vaccine stability indicating analytical methods and their specifications are shown in Table 6.17.

Whole-cell (killed) rCTB finished product release analytical methods and their specifications are shown in Table 6.18.

As per WHO guidelines for the production and control of inactivated oral cholera vaccines (Annex 3, WHO 2004), analytical methods should be appropriately validated.

Table 6.17 Release and shelf life specifications for final lot

Test attribute	Test method	Specification
Sterility	Ph. Eur. direct inoculation	No microbial growth
Identity	Slide agglutination	Agglutination with Inaba and Ogawa specific antibodies
	Ouchterlony	Identity with B subunit (rCTB/CTB)

Table 6.18 Release and shelf life specifications for WCK-rCTB finished product

Test attribute	Test method	Specification
Identity	Slide agglutination	Agglutination with Inaba and Ogawa specific antibodies
Control of labeling	Visual inspection	Labeling in accordance with specifications

6.4.8 Potency/Immunogenicity

At present, there is no animal potency or immunogenicity assay that can be recommended for use as a reliable indicator of the protective efficacy of inactivated oral cholera vaccine in humans or for the detection of subpotent batches. There is as yet no internationally accepted direct method for measuring the potencies of such oral products that guarantees that protective immunity will be elicited in the target population. At present, there is no animal model that can meaningfully be used to measure or predict the potency of these vaccines in humans (Annex 3, WHO 2004). Tables 6.7, 6.8 and 6.9 shows release and shelf life analytical methods for final bulk lots.

6.4.9 General Toxicity Assay

The mouse weight-gain assay in the testing of the cholera whole-cell bulks is aimed at indicating general toxicity as extra precaution in addition to the abnormal toxicity test according to Ph. Eur. This is due to the lack of a meaningful animal model for toxicity testing of oral killed whole-cell cholera vaccine. The assay has, however, not been validated for the purpose of detecting residual CT in the routine testing of the bulks or at the finished product stage which is required according to the WHO requirements. Therefore, the manufacturer has introduced a properly validated GM1 ELISA for routine testing of bulks for the detection of residual CT (EMEA 2005; Annex 3, WHO 2004).

6.4.10 Conclusion

CT is a multifunctional protein that is quite remarkable in many respects. CT was initially thought to be another enterotoxic protein that caused the life-threatening symptoms of cholera, however, numerous studies showed that CT possessed many unique features. Besides its unique structure, mode of intracellular trafficking, and ADP-ribosyltransferase activity. CTB possesses a multifaceted character with regard to functionality. The major contribution of this unique toxin has been in the field of Immunology, functioning as an effective immunogen, adjuvant, or an immunomodulator and holding promise in the area of therapeutics against various types of autoimmune diseases. Moreover, this toxin has a remarkable property for downregulating inflammatory reactions. The molecular mechanisms of its action could open avenues toward development and design of new anti-inflammatory agents for modulating various immune disorders in the foreseeable future (Merritt et al. 1998).

The currently licensed WCK-CTB vaccine has a higher efficacy in children <6 years of age (see Chap. 5 in this book). This vaccine is also considered disadvantageous in terms of high cost and need for cold chain distribution, and leaving room for an improved cholera vaccine for use in developing countries (Lopez et al. 2008; Cumberland 2009). In contrast, WCK without CTB vaccine produced affordably in Vietnam and technology transferred to Shantha Biotechnics (Shanchol, Sanofi Pasteur India) to produce and market internationally (Thiem et al. 2006; Anh at el. 2007; Mahalanabis et al. 2008). This vaccine is similar to Dukoral, except that the formalin-killed classical Ogawa strain Cairo 50 was replaced with strain 569B, in order to increase the amount of the putatively protective TcpA antigen, which is more efficiently expressed by strain 569B (Trach et al. 1997). Two doses of the Vietnam vaccine were shown to be safe, immunogenic, and 66 % protective 8–10 months after immunization for children 1–5 years of age and for older vaccines (Trach et al. 1997).

Though an affordable oral cholera vaccine (WCK without rCTB) has been developed by collaborative efforts from vaccine manufacturers (Vietnam, Shantha Biotechnics, India) and WHO for use in endemic regions, the current challenge for improvement of cholera vaccines during the coming years is the most effective protection of children in developing countries. Children are the most at risk from cholera due to a lack of preexisting immunity (Benenson et al. 1968a, b, c; Mosley et al. 1968; Deen et al. 2008), and a poor vibriocidal response has been observed for children to *V. cholera* vaccines or natural infection (Deen et al. 2008).

There are number of studies that are in progress to develop cholera vaccines with better characteristics of cross-serogroup protection, protection of children, and longer term protective memory (Bishop and Camilli 2011). A number of protein antigen candidates have been investigated using LPS-protein conjugates, outer membrane proteins (OMP), and adsorbed with LPS (Sengupta et al. 1992). Prior studies have shown the presence of anti-El Tor TcpA antibodies in pooled human serum and anti-TcpA seroconversion for 93 % of patients in Bangladesh (Hang et al. 2003; Larocque et al. 2008) and anti-TcpA responses have now been correlated with protection. In vivo-induced antigen technology has also been used to find immunogenic *V. cholerae* proteins expressed in vivo, including the type IV pilus proteins TcpA and PilA (Hang et al. 2003). The *V. cholerae* hemolysin HlyA generates a particularly robust and long-term memory T-cell response, making it a potentially useful immunogen that has not yet been extensively investigated (Weil et al. 2009). Proteomics of stool bacteria, although not yet carried out in a quantitative manner, may reveal insights into antigens expressed in the 'hyperinfectious' state that may also yield candidate protein antigens for inclusion in future cholera vaccines (Larocque et al. 2008). The WHO has issued an updated recommendation that states that vaccination should be used to help control of endemic and epidemic cholera. The availability of an affordable and a safe, feasibly delivered, effective oral vaccine that can be used in resource-limited regions, together with a new pipeline candidate vaccines that may be available in single-dose regimens and long-term protection in children and adults in the future to help fight cholera (WHO 2010; Shin et al. 2011).

Recent insights into correlates of protection, epidemiology and pathogenesis, as well as well characterization of biological vaccine with advance analytical methods, quality and consistent manufacturing may help us design improved vaccines.

6.5 Anthrax Protective Antigen

6.5.1 Introduction to Biodefense Vaccines

Recently, a weapons of mass destruction advisory panel reported to the United States congress that biological agents and not nuclear weapons represent the most significant world threat (A report of the National Biodefense Science Board 2010). Vaccines intended for prophylaxis against potential biological warfare threats (*biothreats*) require special care and procedures because their effectiveness cannot be tested in human challenge studies. They also entail added safety risk and precautions due to handling of the dangerous infectious microbes they target, essential to confirming efficacy in animal models that predict effectiveness in humans. Alternatively, it is difficult to conduct human efficacy clinical trials for new or existing medical countermeasure (MCM) against natural zoonotic infection, such as tularemia, plague, or viral equine encephalitis, because such outbreaks are unpredictable and entail geographical challenges. Instead, licensure in the United States for these MCMs will only be possible using the "Animal Rule" of the FDA, which allows for a demonstration of efficacy in one or more well-characterized animal models which are demonstrated to be relevant to both the disease and its proposed prophylaxis or treatment in humans (Wolfe et al. 2013).

6.5.2 Animal Rule

Vaccine development and ultimate licensure of a Biodefense (BD), countermeasure vaccine is different than that followed for non-BD vaccines. The FDA's Animal Rule was promulgated a decade ago to address the dilemma of not being able to conduct human efficacy trials for biothreats, and its application is based on demonstration in at least two mammal species with evidence of relevance to humans in disease pathology and physiologic/immunological mechanisms of protection (21 CFR 601, 21 CFR 314, FDA 2002 and Also see recent comment periods Federal Register 2009, 2014). This does not mean that studies in two animals is sufficient for a BLA, and may require more, depending on current understanding of the disease and treatment. Thus, testing under the Animal Rule serves as a surrogate for human clinical efficacy studies. Effectiveness must be tested in relevant models and using live, virulent agent. For biothreats of highest military/homeland security concern, the treatment must be protective against organism delivered via the aerosol

mode of exposure. The Animal Rule applies to any new drug designed against biothreats, including vaccines being developed for general use prophylaxis or postexposure prophylaxis, and therapeutics. To clarify some misconceptions, the FDA Animal Rule is not a fast track or accelerated route to licensure, and its purpose is for development of a product for human use, not animal protection. New BD vaccine products still encounter the conventional challenges of a new biological product development, including documentation of methods suitable to define product composition, in process control, release, stability, formulation, safety, and administration. Due to the requirement for demonstrating efficacy against virulent agent, a layer of complexity is added to the standard requirements and for development and validation of analytical methods for any new drug entity. In particular, nonclinical animal studies for potency require that scientists, technicians and operators to maintain top secret security clearance and work within specially designed, licensed, and monitored biological containment facilities. Within these constraints, a candidate must have robust institution sponsorship and programmatic funding to navigate a long and expensive path to a narrow market. Such costs and risks are a principal disincentive to the development of BD vaccine products by industry (Lang and Wood 1999). In response, government agencies have provided the means and funding to help offset these risks and help drive innovation in an effort to supply stockpiles needed for emergency response to biothreats (Matheny et al. 2007).

6.5.3 Anthrax Background

One of several microbial candidates believed to pose great risk as a potential biological weapons threat is the bacterium *B. anthracis*, the cause of the infection anthrax (Wistreich 2001). Anthrax is of particular concern because the infection can occur via inhalation and the etiological agent (spores) is resistant to extreme environmental conditions and so can be weaponized. Awareness, responsiveness, and development of medical countermeasures begun during the Gulf War of the early 1990s (Desert Storm) accelerated after the September 2001 adulteration of the US mail with Anthrax spores. Since then, the United States government has initiated a program to increase awareness, and has established a strategic plan and system for the detection and response to bioterrorist attacks (Wistreich 2001). Humans contract anthrax almost exclusively from contact with, ingestion of, or inhalation of *B. anthracis* spores. Cutaneous anthrax results from a break in the skin and has a mortality rate of about 20 % in untreated cases. Incubation is usually 2–3 days, although it can occur within 12 h and as late as 2 weeks. A small papule appears, followed by a surrounding ring of vesicles about 24 h later. The lesions ulcerate and become black and edematous. In pulmonary anthrax, inhaled spores are carried by macrophages from the lungs to adjacent lymph nodes. The spores germinate, multiply, and cause septicemia. Primary inflammation of the lungs

(pneumonia) may not be detectable (Leppla 2002). Clinical presentation progresses much faster (1–7 days) and mortality is high without immediate antibiotic treatment or prior prophylaxis.

6.5.4 Evolution of the Anthrax Vaccine, Anthrax Vaccine Adsorbed

The existing or *first generation* anthrax vaccine, called Anthrax Vaccine Adsorbed (AVA), originated with studies performed in the 1950s and was first licensed for use in humans in 1970. AVA is produced from culture filtrates of an avirulent, nonencapsulated mutant of the *B. anthracis* Vollum Strain known as V770-NP1-R. AVA, which does not contain *B. anthracis* cells or spores, is a mix of cellular products and contains all three toxic components of lethal factor (LF), edema factor (EF), and protective antigen (PA). However, PA is the principal protein ingredient and primary immune-protective component of AVA (Morb and Mort Weekly Report 2000). The vaccine is now manufactured by Emergent BioSolutions, Inc., USA under the label of BioThrax® AVA (BioThrax® Package Insert—Anthrax Vaccine Adsorbed). To date, more than 11.6 million doses of BioThrax® (Anthrax Vaccine Adsorbed) have been administered to more than 2.9 million individuals. BioThrax® is indicated for the active immunization of individuals between 18 and 65 years of age at high risk of exposure to anthrax. BioThrax® is not licensed for use in a postexposure setting. The safety and efficacy of BioThrax® have not been established in pregnant women, nursing mothers, pediatric populations, or geriatric populations (BioThrax® Package Insert—Anthrax Vaccine Adsorbed).

BioThrax® is a sterile, milky-white suspension for intramuscular injections made from cell-free filtrates of microaerophilic cultures of an avirulent, nonencapsulated strain of *B. anthracis*. The production cultures are grown in a chemically defined protein-free medium consisting of a mixture of amino acids, vitamins, inorganic salts and sugars. The final product, prepared from the sterile filtrate culture fluid contains protein, including the PA protein, released during the growth period, and contains no dead or live bacteria. The final product is formulated to contain 1.2 mg/mL aluminum, added as aluminum hydroxide in 0.85 % sodium chloride. The authors note that elemental aluminum varies among anthrax vaccines, ranging 0.6–2.4 mg/mL in vaccines described below. The final product is formulated to contain 25 μg/mL benzethonium chloride and 100 μg/mL formaldehyde, and added as preservatives (FDA 2012). The Final Drug Product also contains amino acids, vitamins, inorganic salts and sugars (CDC 2012). Some early lots of AVA appeared to contain small amounts of LF and lesser amounts of EF, as determined by induction of antibody responses in the animals, although this has not been reported in human vaccines. No detectable EF was found by Western blotting analysis. ELISA studies found LF to be present in the range of 10–30 ng/mL of fermentation filtrate before aluminum hydroxide adsorption (Joellenbeck et al. 2002). Mouse

macrophage cytotoxicity assay showed that LF is present in a biologically inactive form. Although it is clear that PA by itself is an effective immunogen, it is unknown whether the small amounts of LF or EF that may be present in some lots of the vaccine contribute to protective efficacy (Plotkin et al. 2008).

An FDA approved potency assay for BioThrax® measures survival of vaccinated guinea pigs that are challenged with ~500 LD_{50} of Vollum 1B strain spores injected via intradermal route on the side of the abdomen opposite the vaccination site. Assay details can be found in Gu et al. (2007).

The vaccine is stored at 2–8 °C and is stable for 3 years after a successful potency test results. This vaccine is administered under the skin (subcutaneous, s.c.), rather than directly into the muscle (intramuscular, i.m.). Since subcutaneous administration may be partly responsible for the high number of observed local side effects "(temporary soreness, redness, swelling, itching, and lumps at the injection site in 30 % of male recipients and 60 % of female recipients)," the CDC and collaborators are conducting a human clinical trial of BioThrax® to compare reactogenicity and immunogenicity via the s.c. and i.m. routes of administration. Following the study, the sponsor will present the entire results of the trial to FDA for consideration in elimination of additional doses from the licensed BioThrax® schedule. The sponsor will also supplement clinical data with results from parallel nonhuman primate (NHP) challenge studies plus additional research on immunologic correlates of protection (CDC).

6.5.5 Other Anthrax Licensed Vaccines

The Center for Applied Microbiological Research (Porton Down Salisbury, Wiltshire, UK) developed a similar Anthrax vaccine precipitated by the sterile cell-free culture filtrate of a derivative of the attenuated, unencapsulated Stern stain 34F2 with aluminum potassium sulfate (Hambleton et al. 1984). This vaccine was first administered to humans in the early 1950s and licensed in 1979 (Plotkin et al. 2008). The vaccine contains thimerosal as a preservative. The method to test thimerosal is discussed elsewhere in the chapter (Shrivastaw and Singh 1995a, b). The UK vaccine is administered intramuscular in a regimen of three 0.5 mL doses at 0, 3, and 6 weeks, with a booster dose 6 months after the third dose. Subsequent booster doses are given annually (Turnbull 1991).

A vaccine consisting of a suspension of live spores (similar to Stern strains), named STI-1 for the Sanitary-Technical Institution, has been used for humans in the Soviet Union and its subsequent independent republics since 1953. This vaccine, manufactured by Tblisi Scientific Research Institute of Vaccines and serums (Tblisi, Georgia), the Institute of Microbiology [Kirov (Viatka), Russia Federation], is given by scarification through a 10- to 20-mcL drop of vaccine containing 1.3 to 4×10^8 spores or subcutaneously. The initial dose is followed by a second dose 21 days later, with yearly booster (Plotkin et al. 2008). The Lanzhou Institute of

Biological Products (Lanzhou, Gansu, People's Republic of China) produced in the 1960s another live spore human vaccine given by scarification. This vaccine is based on avirulent strain A16R and a single dose contains 1.6 to 2.4 × 10^8 colony-forming units. A single booster dose is given 6–12 months after the first vaccination (Plotkin et al. 2008).

In the USA, the principal purchasers of the vaccine are the Department of Defense and the Department of Health and Human Services. Ten million doses of AVA have been purchased for the US Strategic National Stockpile for use in the event of a mass bioterrorist attack, used primarily for immunization of troops and defense contractors. However, due to the lengths of time to reach full immunity (18 months for the primary series of 3–6 doses), known reactogenicity, a record of adverse events, and governmental funding for discovery research and product development, there has been renewed interest and effort for the development of an improved anthrax vaccine.

It has been suggested that other antigens in addition to PA would confer improved protection, including those from the spore surface, the vegetative cell capsule and S-layer, as well as LF and ET toxin components (Cote et al. 2012; Kaur and Bhatnagar 2011; Friedlander and Little 2009). These approaches are still early in development. However, as supported by an abundance of basic information plus preclinical and clinical data, the next vaccine after AVA (second generation) will likely be a liquid formulation of rPA combined with an aluminum salt adjuvant. The remainder of this section focuses on the analysis of human anthrax vaccines, including both recombinant PA (rPA) subunit vaccines, as the leading second-generation candidate product form under development, and AVA (BioThrax) for which solutions to efficacy and potency have been pioneered. The current approach from funding agencies and regulatory bodies is that the new rPA vaccines are noninferior to BioThrax.

6.5.6 Pathophysiology and the PA Subunit Approach

Anthrax disease is caused by the gram-positive spore-forming bacterium *B. anthracis*. It is the only member of the genus *Bacillus* capable of causing lethal epidemic disease in humans and other mammals, though closely related *B. cereus* and *B. thuringiensis* species cause human gastroenteritis outbreaks and nosocomial pseudoepidemics (Al-Abir et al. 2011; Jackson et al. 1995). The anthrax toxin is a binary exotoxin comprised from three protein modules: PA functions as a transporter after biding to either lethal factor, forming lethal toxin (LeTx), or to edema factor, forming edema factor (EdTx). Once internalized, LF causes macrophage lysis, immune-system suppression, and death (Agrawal 2004; Friedlander 1986). As a calmodulin-dependent adenylate cyclase, EF stimulates production of the secondary messenger 3′-5′-cyclic adenosine monophosphate (cAMP), contributing to the establishment of infection and anthrax pathophysiology (Liu et al. 2013). Being central to both LeTx and EdTx holotoxins, PA is so named because by itself

it can induce antibodies that protect against both LeTx and EdTx and confer protective immunity against the disease.

PA is a 735 amino acid protein (82,684 kDa), also known as PA83. PA83 binds to a cell surface receptor, where it is cleaved by a furin-like protease, to yield an active 63 kDa fragment (PA63) and an N-terminal 20 kDa fragment (PA20) of unknown function (Hammamieh et al. 2008). Cleavage is essential for toxin action, as PA harboring mutations in the furin cleavage site is completely nontoxic to murine macrophages and devoid of pathogenic effects in vivo in sensitive mice when reconstituted with LF (Brey 2005). Current understanding models effector-bound PA63 holotoxin as an octomer (e.g., PA_8LF_4), which is endocytosed and then translocates effector (e.g., LF) into the acidified endosome (Feld et al. 2010). Although the key targets LT and ET causing host lethality are still unknown, it is believed that pathophysiology results from their coordinately damage to two distinct vital systems composed of myeloid cells and hepatocytes (Liu et al. 2013; Cote et al. 2011). As PA is essential to the function of both toxins and anthrax pathophysiology, PA is the proposed primary immunogen for an improved human anthrax vaccine against the biothreat posed by *B. anthracis* spores.

The subunit PA anthrax vaccine has been shown in numerous animal studies to confer protective immunity against lethal aerosol challenge (Little et al. 2006, 2007; Williamson et al. 2005; Laird et al. 2004). A variety of adjuvants have been shown to be effective for inducing protective immunity of PA formulations in animal spore challenge studies, including aluminum salts, Saponin Monophsophoryl lipid A, and spore antigens (Ivins et al. 1998; Berthold et al. 2005; Cote et al. 2012; Peachman et al. 2012). Nevertheless, the aluminum hydroxide salts of alum (aluminum hydroxide gel) or Alhydrogel® (aluminum oxyhydroxide gel) are the adjuvants used in the currently licensed vaccine and the leading second-generation anthrax vaccine candidate products. While the choice of these formulations is based on ample prior testing and evidence, the deposition of a protein antigen active ingredient onto an aluminum salt presents challenges for the design and use of stability methods, as is discussed further below.

6.5.7 Recombinant PA (RPA)

The first rPA was expressed in, and purified from, the supernatant of a nonsporogenic, avirulent *B. anthracis* strain (Ivins et al. 1995, 1998; Farchaus et al. 1998). Substantial effort was given to reducing fragments observed as a major source of impurities (Rhie et al. 2005; Jendrek et al. 2003; Ramirez et al. 2002; Miller et al. 1998). Other popular derivations express and produce rPA in *E. coli* (Brown et al. 2010; Laird et al. 2004; Gwinn et al. 2006; Williamson et al. 2005), as pioneered by demonstration of unusually pure rPA protein from inclusion bodies (Gupta et al. 1999). In one of these processes, soluble rPA is expressed in relatively high amounts in the periplasm of *E. coli* from shake flasks and bioreactors and purified using Q-Sepharose-HP and hydroxyapatite chromatography, and routinely found to

be 96–98 % pure. Yields of purified PA varied depending on the method of production; however, medium cell density fermentations resulted in approximately 370 mg/L of highly pure biologically active PA protein. These results exhibit the ability to generate gram quantities of PA from *E. coli*.

Following is a discussion of the analytical approaches used in the development of rPA anthrax vaccines. Some of these methods have translated for use as release assays.

6.5.8 Analysis of the Recombinant Anthrax Protective Antigen

6.5.8.1 Animal Survival Models and Protective Immunity

Protective immunity is accessed via animal survival studies against an aerosol challenge of *B. anthracis* spores. A number of animal models have been used including mice, guinea pig, rabbit, and up to NHP models. An FDA advisory meeting report has indicted that both the NHP and the New Zealand white (NZW) rabbit are considered animal models of choice to generate pivotal animal survival data for assessing the efficacy of a PA-based vaccine (FDA 2010). Animals are vaccinated and then challenged with a targeted dose of LD_{50} spores from the Ames isolate of *B. anthracis* and monitored for survival. The LD_{50} of Ames spores in NZW rabbits is 1.1×10^5 spores (Pitt et al. 2001). Since survival data alone is no longer sufficient, a primary goal of such studies is to define antibody response in a given animal model for developing a serological correlate of protective immunity, as described next. Concentration of protective antibody can be demonstrated as a correlate of protection if an increase in the amount of inoculated vaccine immunogen produces increased survival after challenge and increased antibody response. A correlate of protective immunity against anthrax has been identified for NZW rabbits immunized with a recombinant PA (rPA) vaccine formulated with aluminum hydroxide adjuvant (Alhydrogel) (Little et al. 2004a, b; Pitt et al. 2001). Due to the presence of PA as principal immune component, animal models and potency tests for AVA (BioThrax) have served as the benchmark for development and regulatory oversight of similar requirements for rPA subunit vaccines. Human protection obviously cannot be test; the approach is to compare human serology to a comparative, *Human Equivalent*, response in a model animal. This also applies to the notion of potency.

6.5.8.2 Potency Assays

Due to considerations of animal welfare, assay variability, plus the high cost and security requirements for handling biological threat agent such as viable anthrax

spores, the FDA and NIH have issued goals to replace assays of lethal animal challenge. As a result, immunogenicity assays are developed as potency tests and serological surrogate of protection. Two potency assays for anthrax vaccines have centered on measuring the activity or concentration of specific antibodies, based either on quantitative anti-rPA IgG ELISA or on macrophage lysis tests known as toxin neutralizing antibody assay (TNA). Both have been compared and serological correlates of protection for each have been established in rabbits for a recombinant anthrax vaccine (Parreiras et al. 2009; Little et al. 2004a, b; Pitt et al. 2001). An FDA effort to justify either the ELISA or TNA approach for potency measurement has indicated that the ELISA may not be as reliable (Brady et al. 2010). A TNA has been validated that measures the functional ability of antisera to specifically protect J774A.1 cells against *B. anthracis* LeTx cytotoxicity using human and rabbit antisera produced against the AVA vaccine (Hering et al. 2004). An important additional concern for potency testing is the maintenance of recombinant LF protein (rLF) and macrophage cell lines as critical reagents for TNA-based assays.

6.5.8.3 Biophysical Methods for Lot Release

Although specific lot release strategies are proprietary for rPA products in development, the general approach follows FDA guidelines for API release and DS release, including tests for identity, strength, purity, and potency of API, residual concentrations (antibiotics, endotoxin) strength of excipients, preservatives, stabilizers, and adjuvants.

The purity of the PA product has been characterized by reversed-phase high-pressure liquid chromatography (Farchaus et al. 1998), sodium dodecyl sulfate (SDS)-capillary electrophoresis, capillary isoelectric focusing, native gel electrophoresis, and SDS-polyacrylamide gel electrophoresis (PAGE). Methods for rPA identity, quality, strength, and structural characterization have included PAGE, Ion exchange chromatography (IEX), isoelectric focusing IEF/cIEF, RP-HPLC, HP-SEC, peptide mapping (LC/MS), circular dichroism (CD), differential scanning calorimetry (DSC), intrinsic fluorescence (Wagner et al. 2012; Soliakov et al. 2012), and light scattering.

6.5.8.4 Stability

While a standard ensemble of release and stability tests applies to rPA for insuring consistency in purity, strength, and quality, cumulative evidence has indicated that deamidation is the principle stability issue confronting rPA as API and formulated DP (Verma et al. 2013; D'Souza et al. 2013; Powell et al. 2007; Ribot et al. 2006; Zomber et al. 2005). Figure 6.27 illustrates correlation between the progression of charged isoforms, loss of biological activity, and increased deamidation at specific sites (Powell et al. 2007). Fragmentation, oxidation, and aggregation are also associated with rPA degradation though to lesser extents than deamidation.

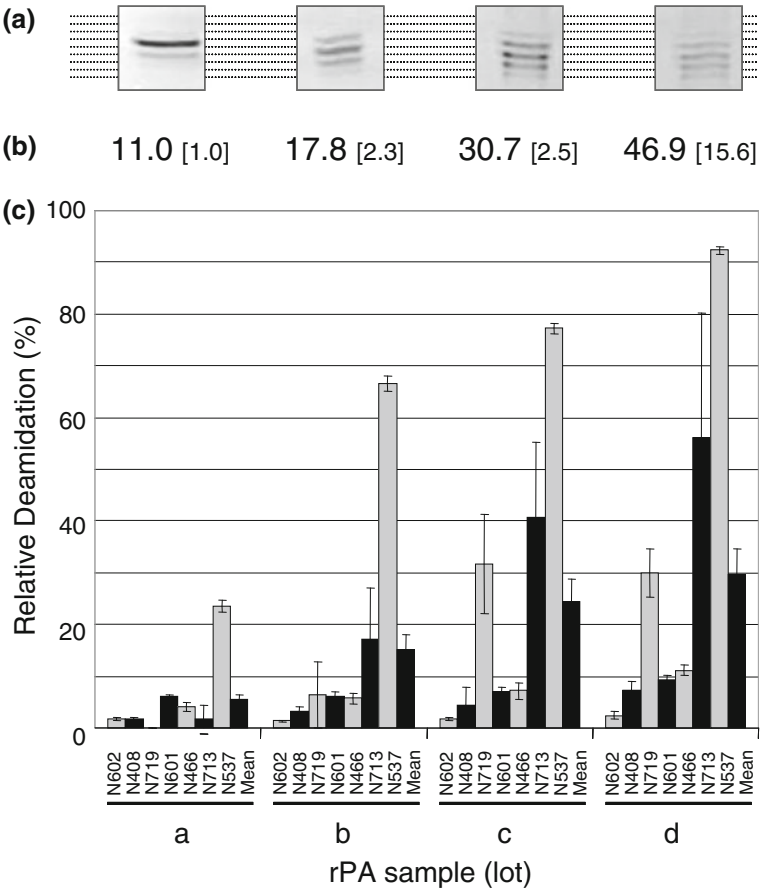


Fig. 6.27 Multiple-site deamidation and decreased biological activity correlate with increased isoform complexity of different rPA preparations. Comparison of isoform complexity and biological activity with percent deamidation at multiple Asn sites in different rPA preparations. **A** Native PhastGels of whole PA protein preparations as designated below. Horizontal guidelines aid in comparing vertical positions of protein isoforms between gels. **B** Cytotoxic activities of same PA preparations given in EC50 (ng/mL) with standard deviation in brackets. **C** Relative deamidation for seven Asn residues in each protein preparation (abscissa) expressed as percent deamidated (ordinate). Samples, *vertically* aligned with gel images in A, comprise: *a* List Biologicals; *b* R&D rPA lot 121096a; *c* R&D rPA lot 121096b; *d* rPA lot 022795b. *Residues* (1) N602; (2) N408; (3) N719; (4) N601; (5) N466; (6) N713; (7) N537; *M* mean. The figure is adopted from Powell et al. (2007) and reproduced with permission from John Wiley and Son

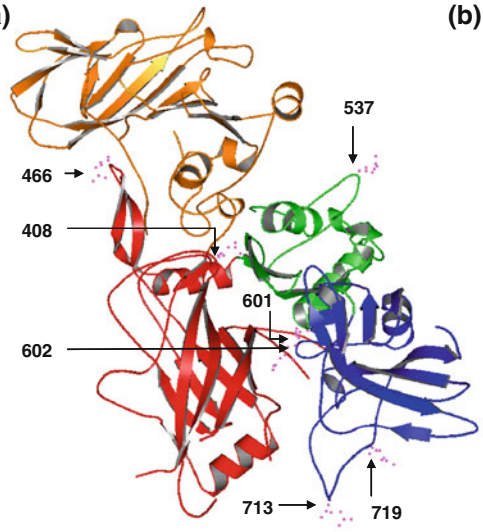
Fragmentation has largely been resolved with preparations of higher purity, including expression in *E. coli* and improved purification schemes, as noted above. Structural properties and quality attributes of rPA in these respects have been measured by a variety of methods which require supplementary preanalytical techniques to desorb protein from the adjuvant matrix in formulated product, as discussed below.

6.5.8.5 Quality

As discussed, charge heterogeneity is a principal concern for rPA release and stability. Charge isoforms in the anthrax toxin proteins were noted since their first isolation and this phenomenon is now known to be caused by deamidation at specific asparagines (Powell et al. 2007). Of the PAGE methods employed, the native PhastGel (GE Healthcare) provides a rapid and simple analysis for screening rPA charge isoforms (Ribot et al. 2006; Powell et al. 2007). Standard and two-dimensional PAGE provide better separation for analysis of whole protein and isoforms (Powell et al. 2007; Zomber et al. 2005). While anion exchange chromatography (IEX) has been used to isolate isoforms for structural and functional study (Ribot et al. 2006), isoelectric focusing (IEF) in gel and capillary format (cIEF) have been useful in monitoring rPA charge heterogeneity (Ribot et al. 2006; Powell et al. 2007; Zomber et al. 2005). Peptide mapping and amino acid sequencing by mass spectrometry (LC/MS and LC-MS/MS) enable the direct connection between charge heterogeneity and deamidation, as noted above (Verma et al. 2013; D'Souza et al. 2013; Powell et al. 2007; Zomber et al. 2005). Figure 6.2 shows a ribbon structure depiction of rPA and the most labile asparagines (Powell et al. 2007). An important general finding from this work, applicable to all protein products intended for use in humans, is the demonstration that computer algorithms for predicting deamidation are not yet sufficiently accurate to eliminate deamidation as potential route of product degradation. Such models are built on peptide data, and proteins deamidate differentially as has been shown by empirical evidence (D'Souza et al. 2013; Powell et al. 2007; Zomber et al. 2005). For example, in rPA, AsnN537 showed the highest percent of deamidation at all stages of purification tested, although its predicted rate of deamidation ranked 10th by, and alternatively, other asparagines of higher predicted rates were unmodified (Fig. 6.28).

Direct correlation between isoform complexity and percent deamidation has been observed such that each decreased with purity and increased with protein aging or forced stress (Powell et al. 2007; D'Souza et al. 2013). rPA with more isoforms and greater deamidation displayed lower activities for furin cleavage, heptamerization, and holotoxin formation (Powell et al. 2007) and has been confirmed in other rPA products both as pure protein (Zomber et al. 2005) or bound to adjuvant (D'Souza et al. 2013), including observation that ASN 713 and 719 deamidation rapidly when rPA is adjuvanted on Alhydrogel® (D'Souza et al. 2013). To further explore whether deamidation of rPA affects vaccine immunogenicity, recombinant "genetically deamidated" forms of rPA using site-directed mutagenesis that replaced six deamidation-prone asparagine residues with aspartate, glutamine, or alanine. While the structure and biological function of the six-Asp mutant rPA was not significantly altered relative to the wild-type rPA protein product, its immunogenicity as an aluminum-adjuvanted vaccine was significantly lower than the wild-type rPA vaccine formulation by TNA (Verma et al. 2013). This confirmed that specific deamidation of rPA adversely affects the immunogenicity of rPA, and suggests that concurrent deamidation six sites may be a threshold for such effect.

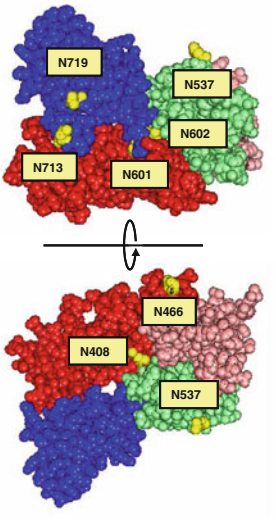
(a)



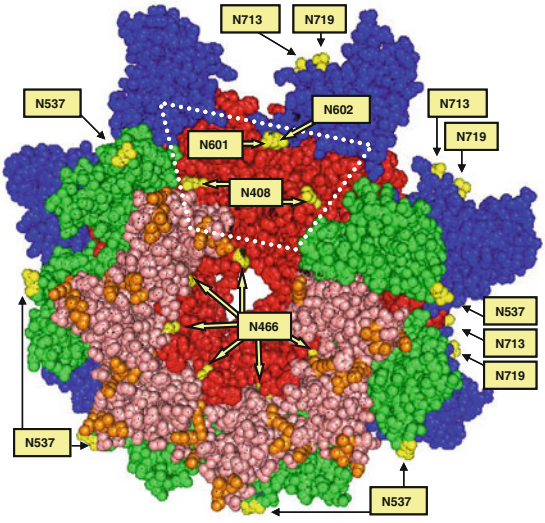
(b)

Asn Site	$t_{1/2}$ (days)	Rank Order		
		P	F	T
713	24	1st	2nd	2nd
162	38	2nd	0	0
719	49	3rd	5th	3rd
306	53	4th	0	0
010	57	5th	0	0
102	67	6th	0	0
006	81	7th	0	0
281	117	8th	0	0
466	133	9th	3th	4th
537	219	10th	1st	1st
408	4363	37th	6th	6th
601	48150	57th	4th	5th
602	2,594,300	68th	7th	7th

(c)



(d)



◀ **Fig. 6.28** Comparison of computational and observed deamidation in rPA. **a** Table comparing rank order of observed Asn deamidation to that predicted by rate at www.deamidation.org using the protein database identifier for PA (1ACC). Abbreviations as follows: Asn site, amino acid coordinate of Asn as defined in Fig. 6.2. t1/2, half-life in days of deamidation calculated from the theoretical coefficient of deamidation; rank order for propensity of deamidation as listed by: *P* predicted rate calculated by the Robinson algorithm; *F* observed percentage for fresh PA protein; *T* observed percentage for treated PA protein; 0 no deamidation observed; **b** Projection of seven observed sites of deamidation onto the three-dimensional structure of PA. *Arrows mark* deamidated Asp with numeric designation by coordinate number, as defined in Fig. 6.5; *pink dots* projection of original asparginyl side group. Color scheme as follows: *domain 1* (*orange*, residues 1–258) contains two calcium-binding sites, the cleavage site for furin class proteases, and the binding site for EF and lethal LF. *Domain 2* (*red*, 259–487) forms most of the channel and protein–protein interactions. *Domain 3* (*green*, 488–595) may play a role in oligomerization. *Domain 4* (*blue*, 596–735) is responsible for binding the cell surface receptor; **c** following page, Projection of seven observed Asn deamidation sites onto a space-filling model of the X-ray structure of monomeric PA, and rotated 1808 around its long axis for full viewing. Color scheme as for panel B except domain 1 is *pink*; **d** projection of seven observed Asn deamidation sites onto the modeled structure of PA heptameric prepore (PA63)7, central pore axis angled slightly *down* and *left*, and one LF-binding domain (*pink*) removed (*dotted trapezoid*), to expose buried sites and position others for viewing. Deamidation at residue N537 is highest among all samples tested, while deamidation at residues N408 and N466 statistically correlate with loss of PA biological activity. Same coloration as in panel C except LF/EF factor binding sites are shown in *orange*. The figure is adopted from Powell et al. (2007) and reproduced with permission from John Wiley and Son

6.5.9 Adjuvanted Formulated Vaccine

As a formulated drug product, rPA is combined with an adjuvant since it is not protective by itself, and aluminum salts such as Alhydrogel® are used in current development of second-generation anthrax vaccines. In order to apply structural assays developed on soluble rPA, the methods must be modified, first to remove (desorb) protein from the adjuvant and then to adjust acceptance criteria. Desorption methods must strip protein from the aluminum depot, in sufficient amount and without altering quality, and then leave it in a matrix suitable for analysis. Assay specifications and acceptance need to be reestablished since some alteration occurs as protein is bound to and then removed from aluminum salts.

Bound rPA can be desorbed from Alhydrogel by 18-h exposure to 25 mM phosphate ion to yield test material that confirms to rPA standard by SDS-PAGE and HP-SEC (Jendrek et al. 2003). Recovery drops from 75 to 12 % in the presence of 10 mM EDTA. Analysis since then has shown that the native structure of rPA, which binds two moles of 2 mol of calcium in Domain 1 per mole of protein, is not disrupted by adjuvanting and that Alhydrogel does not bind residual calcium (Soliakov et al. 2012). Other successful methods have been developed for desorption, including the use of chaotropic agents or detergents for subsequent analysis by SDS-PAGE, RP-HPLC, cIEF, and LC-MS.

To circumvent the complications of desorbing protein prior to analysis, in situ methods have been developed to characterize rPA structure as bound to Alhydrogel®. There is a concern that structural and functional properties of rPA may alter

after long-term adsorption to aluminum adjuvants, leading to instability of quality and efficacy (Wagner et al. 2012). Several studies describe dynamic changes in rPA structure and immunogenicity while it is adsorbed to Alhydrogel® (Wagner et al. 2012; Solaikov et al. 2012; Hu et al. 2012; Ganessan et al. 2012; Watkinson et al. 2013), and deamidation appears to increase with contact of rPA to Alhydrogel® (D'Souza et al. 2013). As yet, there is no direct connection between changes in secondary structure and loss of protection against a lethal challenge. Nevertheless, aluminum adjuvants including Alhydrogel® clearly increase the stability of thawed rPA protein in vitro and in vivo (Watkinson et al. 2013).

6.6 Conclusions

Recombinant bacterial subunit vaccines are 1. Purified surface and secreted proteins, 2. Genetically Detoxified virulence factors, 3. Mutated chemical proteins, and 4. Polysaccharide-protein complexes. These are made by manipulating bacterial pathogen's reverse vaccinology and structural vaccinology and cloning and expression in heterologous host and thereby expressing and purifying these recombinant bacterial subunit vaccines. These subunit vaccines offer few major advantages and disadvantages (Unnikrishnan et al. 2012), which can be summarized as follows:

Advantages	Disadvantages
• Well-characterized products	• Manufacturing process highly complex and demanding
• Increased safety and efficacy	• Usually need proper adjuvants
• Only the useful and more potent antigens can be included in the vaccine	• Subjected to post-translational modification and not guaranteed in a heterologous system

Subunit vaccines, containing specific antigens or their respective genes from pathogenic organisms, are regarded as one of the safest, although their efficacy is lower than that observed for vaccines consisting of whole, attenuated cells. To increase efficacy, one strategy would be to obtain a universal vaccine against many isolates of specific bacterial species is to include more than one antigen into the vaccine prototype as noted above. And the other way to enhance and/or modulate the host immune response is to deliver a vaccine with an appropriate adjuvant. The tremendous progress of proteomics during the past few years has contributed to every successive stage of antigen characterization (Adamczyk-Poplawska et al. 2011).

In summary, recombinant DNA technology has enabled the rationale design of vaccines against complex human diseases, including of subunit vaccines as described in this chapter. Despite excellent evidence of success of recombinant vaccines in preclinical and clinical studies, few recombinant vaccines are licensed for human use. This can however be attributed to the intense regulatory process

revolving around recombinant vaccine production. In the case of medical countermeasures against biological threats such as anthrax, there are additional rules and regulations including adherence to biosecurity licensing and oversight plus demonstration of proposed human efficacy via the Animal Rule. Improved adjuvant technologies, new approaches like systems biology, proteomics and genomics, and rapidly advancing technologies for enhanced manipulation of humoral, cellular, and innate immunity will all play a role in driving the next generation of protein subunit vaccines (Unnikrishnan et al. 2012). Finally, with the advent of the first synthetic bacterium, applications of synthetic biology in vaccine design will be the most rational approach in the years to come.

Acknowledgments The author is grateful to Dr. Juan Arciniega at the US FDA for reviewing and editing the D, T, aP, and DTaP sections. The authors are grateful to Eleonora Fregolino, Luca Simeone, Chiara Parlati, Alessandro Vadi, Rosy Galasso, Cristiana Campa for their important contributions to OMV understanding, and to Anna Rita Taddei, for producing the TEM data reported, within the Novartis Vaccines collaboration with C.I.M.E. (Centro Interdipartimentale Microscopia Elettronica), University of Tuscia, Viterbo, Italy.

References

A Report of the National Biodefense Science Board (2010) Where are the countermeasures? Protecting America's health from CBRN threats

Adamczyk-Poplawska M, Markowicz S, Jagusztyn-Krynicka EK (2011) Proteomics for development of vaccines. J Proteomics 74:2596–2616

Aggerback H, Heron I (1991) Improvement of a vero cell assay to determine diphtheria antitoxin content in sera. Biologicals 19:71–76

Agrawal A, Pulendran B (2004) Anthrax lethal toxin: a weapon of multisystem destruction cell. Mol Life Sci 61:2859–2865

Al-Abri SS, Al-Jardani AK, Al-Hosni MS, Kurup PJ, Al-Busaidi S, Beeching NJ (2011) A hospital acquired outbreak of *Bacillus cereus* gastroenteritis, Oman. J Infect Public Health 4 (4):180–186

Albert MJ, Alam K, Rahman AS, Huda S, Sack RB (1994) Lack of cross protection against diarrhea due to *Vibrio cholera* O1 after oral immunization of rabbits with *V. cholera* O139 Bengal. J Infect Dis 169:709–710

Alexander C, Rietschel ET (2001) Bacterial Lipopolysaccharide and innate immunity. Endotoxin Res 7:167–202

Alonso MJ, Gupta RK, Min C, Siber GR, Langer R (1994) Biodegradable microspheres as controlled-release tetanus toxoid delivery systems. Vaccines 12:299–306

Anh DD, Canh do G, Lopez AL, Thiem VD, Long PT, Son NH, Deen J, von Seidlein L, Carbis R, Han SH, Shin SH, Attridge S, Holmgren J, Clemens J (2007) Safety and immunogenicity of a reformulated Vietnamese bivalent killed, whole-cell, oral cholera vaccine in adults. Vaccine 25 (6):1149–1155

Arciniega JL, Corvette L, Hsu H, Lynn F, Romani T, Dobbelaer R (2011) Target alternative vaccine safety testing strategies for pertussis toxin. Procedia Vaccinol 5:248–260

Asaduzzaman M, Ryan ET, John M, Hang L, Khan A, Faruque AS, Taylor RK, Calderwood SB, Qadri F (2004) The major subunit of the toxin-coregulated pilus TcpA induces mucosal and systemic immunoglobulin a immune responses in patients with cholera caused by *Vibrio cholerae* O1 and O139. Infect Immun 72(8):4448–4454

Bache C, Hoonakker M, Hendriksen C, Buchheit K-H, Spreitzer I, Montag T (2012) Workshop on animal free detection of pertussis toxin in vaccines-alternatives to the histamine sensitization test. Biologicals 40:309–311

Benenson AS, Saad A, Paul M (1968a) Serological studies in cholera. I. Vibrio agglutinin response of cholera patients determined by a microtechnique. Bull World Health Organ. 38 (2):267–276

Benenson AS, Saad A, Mosley WH (1968b) Serological studies in cholera. 2. The vibriocidal antibody response of cholera patients determined by a microtechnique. Bull World Health Organ 38(2):277–285

Benenson AS, Saad A, Mosley WH, Ahmed A (1968c) Serological studies in cholera. 3. Serum toxin neutralization–rise in titre in response to infection with Vibrio cholerae, and the level in the "normal" population of East Pakistan. Bull World Health Organ 38(2):287–295

Bergfors E, Trollfors B, Inerot A (2003) Unexpectedly high incidence of persistent itching nodules and delayed hypersensitivity to aluminum in children after the use of adsorbed vaccines from a single manufacturer. Vaccine 22:64–69

Berthold I, Pombo M-L, Wagner L, Arcinieg JL (2005) Immunogenicity in mice of anthrax recombinant protective antigen in the presence of aluminum adjuvants. Vaccine 23:1993–1999

Bishop AL, Camilli A (2011) A. *Vibrio cholera*: lessons for mucosal vaccine design. Expert Rev Vaccine 10(1):79–94

Brady RA, Verma A, Meade BD, Burns DL (2010) Analysis of antibody responses to protective antigen-based anthrax vaccines through use of competitive assays. Clin Vaccine Immunol 9:1390–1397

Brey RN (2005) Molecular basis for improved anthrax vaccines. Adv Drug Deliv Rev 57 (9):1266–1292

CDC (2012) Epidemiology and prevention of vaccine-preventable diseases. The pink book: course textbook, 12th Edn. Appendix B vaccine excipient & media summary. http://www.cdc.gov/vaccines/pubs/pinkbook/downloads/appendices/B/excipient-table-2.pdf

FDA (2010) Pathway to licensure for protective antigen-based anthrax vaccines for a post-exposure prophylaxis indication using the animal rule. http://www.fda.gov/advisorycommittees/committeesmeetingmaterials/bloodvaccinesandotherbiologics/vaccinesandrelated biologicalproductsadvisorycommittee/ucm197728.htm

Brown BK, Cox J, Gillis A, VanCott TC, Marovich M, Milazzo M, Antonille TS, Wieczorek L, McKee KT Jr, Metcalfe K, Mallory RM, Birx D, Polonis VR, Robb ML (2010) Phase I study of safety and immunogenicity of an *Escherichia coli*-derived recombinant protective antigen (rPA) vaccine to prevent anthrax in adults. PLoS ONE 5(11):e13849

Burns DL, Kenimer JG, Manclark CR (1987) Role of the A subunit of pertussis toxin in alteration of Chinese hamster ovary cell morphology. Infect Immun 55:24–28

Tondella ML, Carlone, GM, Messonnier N, Quinn CP, Meade BD, Burns DL, Cherry JD, Guiso N, Hewlett EL, Edwards KM, Xing D, Giammanco A, Wirsing von Konig CH, Han L, Hueston L, Robbins JB, Powell M, Mink CM, Poolman JT, Hildreth SW, Lynn F, Morris A (2009) International *Bordetella pertussis* assay standardization and harmonization meeting report. Vaccine 27:803–814

Carroll SF, Barbieri JT, Collier J (1988) Diphtheria toxin: purification and properties. In: Harshman S (ed) Methods in enzymology, vol 165. Academic press, New York, pp 68–76

Chitnis DS, Sharma KD, Kamat RS (1982) Role of bacterial adhesion in the pathogenesis of cholera. J Med Microbiol 15(1):43–51

Cholera Working Group ICDDR, Bangladesh (1993) Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholera* O139 synonym Bengal. Lancet 342(8868):387–390

Clemens J, Jertborn M, Sack D, Stanton B, Holmgren J, Khan MR, Huda S (1986) Effect of neutralization of gastric acid on immune responses to oral B subunit killed whole-cell cholera vaccine. J Infect Dis 154:175–178

CDC-NCID-3344, ClinicalTrials identifier NCT00119067. www.clinicaltrials.gov

Cookson BT, Cho H-L, Herwaldt LA, Goldman WE (1989) Biological activities and chemical composition of purified tracheal cytotoxin of *Bordetella pertussis*. Infect Immun 57:2223–2229

Coombes L, Stickings P, Tierney R, Rigsby P, Sesardic D (2009) Development and use of a novel in vitro assay for testing of diphtheria toxoid in combination vaccines. J Immunol Methods 350:142–149

Coombes L, Tierney R, Rigsby P, Sesardic D, Stickings P (2012) In vitro antigen ELISA for quality control of tetanus vaccines. Biologicals 40:466–472

Corbel MJ, Xing DKL (2004) Toxicity and potency evaluation of pertussis vaccines. Expert Rev Vaccines 3:89–101

Corbel MJ, Xing DKL, Bolgiano B, Hockley DJ (1999) Approaches to the control of a cellular pertussis vaccines. Biologicals 27:133–141

Cote CK, Welkos SL, Bozue J (2011) Key aspects of the molecular and cellular basis of inhalational anthrax. Microbes Infect 13(14–15):1146–1155

Cote CK, Kaatz L, Reinhardt J, Bozue J, Tobery SA, Bassett AD, Sanz P, Darnell SC, Alem F, O'Brien AD, Welkos SL (2012) Characterization of a multi-component anthrax vaccine designed to target the initial stages of infection as well as toxaemia. J Med Microbiol 61:1380–1392

Cumberland S (2009) An old enemy returns. Bull World Health Organ 87(2):85–86

Cyr T, Menzies AJ, Calver J, Whitehouse LW (2001) A quantitative analysis for the ADP-Ribosylation activity of pertussis toxin: an enzymatic-HPLC coupled assay applicable to formulated whole cell and acellular pertussis vaccine products. Biologicals 29:81–95

Dahneke BE (1983) Measurement of suspended particles by quasielastic light scattering. Wiley, New York, p 570

Dakterzada F, Mobazer AM, Roudkenar M, Forouzandeh M (2012) Production of pentameric cholera toxin B subunit in *Escherichia coli*. Avicenna J Med Biotechnol 4(2):89–94

Deen JL, Von Seidlein L, Sur D, Agtini M, Lucas MES, Lopez AL, Kim DR, Ali M, Clemens JD (2008) The high burden of cholera in children: comparison of incidence from endemic areas in Asia and Africa. PLoS Negl Trop Dis 2(2):e173

Del Giudice G, Rappuoli R (1999) Molecular approaches for safer and stronger vaccines. Schweiz Med Wochenschr 129(46):1744–1748

Del Giudice G, Pizza M, Rappuoli R, (1998) Molecular basis of vaccination. Mol Aspects Med 19 (1):1–70

D'Souza AJ, Mar KD, Huang J, Majumdar S, Ford BM, Dyas B, Ulrich RG, Sullivan VJ (2013) Rapid deamidation of recombinant protective antigen when adsorbed on aluminum hydroxide gel correlates with reduced potency of vaccine. J Pharm Sci 102(2):454–461

Dukoral Package Leaflet (2013) Information for the user. Crucell Sweden AB, 105 21 Stockholm, Sweden

EMEA (2005) Scientific discussion module for the approval of Dukoral

European Pharmacopoeia (2014) 8.2 edition

Farchaus JW, Ribot WJ, Jendrek S, Little SF (1998) Fermentation, purification, and characterization of protective antigen from a recombinant, a virulent strain of *Bacillus anthracis*. Appl Environ Microbiol 64(3):982–991

FDA (2002) Approval of biological products when human efficacy studies are not ethical or feasible. 21 CFR 601 subpart H (for biological products) 21 CFR 314 subpart I for new drugs (for drugs)

FDA (2012) BioThrax®-Package Insert. http://www.fda.gov/downloads/BiologicsBloodVaccines/BloodBloodProducts/ApprovedProducts/LicensedProductsBLAs/UCM074923.pdf

Feavers I, Griffiths E, Baca-Estrada M, Knezevic I, Zhou T (2012) WHO/Health Canada meeting on regulatory considerations for evaluation and licensing of new meningococcal group B vaccines, Ottawa Canada 3–4 October 2011. Biologicals 40(6):507–516

Federal Register (2005) Biological products: bacterial vaccines and toxoids; implementation of efficacy review, vol 70, pp 75018–75028

Federal Register (2009) Draft guidance for industry on animal models–essential elements to address efficacy under the animal rule. Notices 74(12):3610–3611

Federal Register (2014) Product development under the animal rule, revised draft guidance for industry; availability, vol 79, no 106, pp 31950–31951

Feld GK, Thoren KL, Kintzer AF, Sterling HJ, Tang LI, Greenberg SG, Williams ER, Krantz BA (2010) Structural basis for the unfolding of anthrax lethal factor by protective antigen oligomers. Nat Struct Mol Biol 17(11):1383–1390

Ferrari G, Garaguso I, Adu-Bobie J, Doro F, Taddei AR, Biolchi A, Brunelli B, Giuliani MM, Pizza M, Norais N, Grandi G (2006) Proteomics 6:1856–1866

Frasch CE, van Alphen L, Holst J, Poolman J, Rosenqvist E (2001) Preparation of outer membrane protein vaccines against meningococcal disease. In: Pollard AJ, Maiden MCJ (eds) Methods in molecular medicine, vol 66. Meningococcal disease protocols. Humana Press Inc, Totowa

Fredriksen JH, Rosenqvist E, Wedege E, Bryn K, Bjune G, Frøholm LO, Lindbak AK, Møgster B, Namork E, Rye U (1991) NIPH Ann 14(2):67–79 (discussion 79–80)

Friedlander AM (1986) Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. J Biol Chem 261:7123–7126

Friedlander AM, Little SF (2009) Advances in the development of next-generation anthrax vaccines. Vaccine 27(Suppl 4):D28–D32

Gaines-Das R, Horiuchi Y, Zhang SM, Newland P, Kim Y, Corbel M, Xing D (2009) Modified intra-cerebral challenge assay for acellular pertussis vaccines: comparisons among whole cell and acellular vaccines. Vaccine 27:6824–6832

Galazka AM (1993) The immunological basis for immunization series, module 3: tetanus. Global programme for vaccines and immunization expanded programme on immunization, WHO, Geneva, Switzerland

Ganesan A, Watkinson A, Moore BD (2012) Biophysical characterization of thermal-induced precipitates of recombinant anthrax protective antigen: evidence for kinetically trapped unfolding domains in solid-state. Eur J Pharm Biopharm 82(3):475–484

Gentschev I, Dietrich G, Spreng S, Kolb-Mäurer A, Brinkmann V, Grode L, Hess J, Kaufmann SH, Goebel W (2001) Recombinant attenuated bacteria for the delivery of subunit vaccines. Vaccine 19:2621–2628

Glenny AT, Hopkins BE (1923) Diphtheria toxoid as an immunising agent. Br J Exp Pathol 4:283–288

Gu M, Hine PM, Jackson WJ, Giri L, Nabors GS (2007) Increased potency of BioThrax® anthrax vaccine with the addition of the C-class CpG oligonucleotide adjuvant CPG 10109. Vaccine 25:526–534

Gupta P, Waheed SM, Bhatnagar R (1999) Expression and purification of the recombinant protective antigen of *Bacillus anthracis*. Protein Expr Purif 16(3):369–376

Gwinn W, Zhang M, Mon S, Sampey D, Zukauskas D, Kassebaum C, Zmuda JF, Tsai A, Laird MW (2006) Scalable purification of *Bacillus anthracis* protective antigen from *Escherichia coli*. Protein Expr Purif 45(1):30–36

Hambleton P, Carman JA, Melling J (1984) Anthrax: the disease in relation to vaccines 2:125–132

Hammamieh R, Ribot WJ, Abshire TG, Jett M, Ezzell J (2008) Activity of the *Bacillus anthracis* 20 kDa protective antigen component. BMC Infect Dis 8:124

Hang L, John M, Asaduzzaman M, Bridges EA, Vanderspurt C, Kirn TJ, Taylor RK, Hillman JD, Proguiske-Fox A, Handfield M, Ryan ET, Calderwood SB (2003) Use of in vivo-induced antigen technology (IVIAT) to identify genes uniquely expressed during human infection with *Vibrio cholerae*. Proc Natl Acad Sci USA 100(14):8508–8513

Hering D, Thompson W, Hewetson J, Little S, Norris S, Pace-Templeton J (2004) Validation of the anthrax lethal toxin neutralization assay. Biologicals 32:17–27

Herrington DA, Hall RH, Losonsky G, Mekalanos JJ, Taylor RK, Levine MM (1988) Toxin, toxin-coregulated pili, and the toxR regulon are essential for Vibrio cholerae pathogenesis in humans. J Exp Med 168(4):1487–1492

Hewlett EL, Sauer KT, Myers GA, Cowell JL, Guerrant RL (1983) Induction of a novel morphological response in Chinese hamster ovary cells by pertussis toxin. Infect immun 40:1198–1203

Hilleman MR (2000) Vaccines in historic evolution and perspective: a narrative of vaccine discoveries. Vaccine 18:1436–1447

Thiem VD, Deen JL, Von Seidlein L, Canh do G, Anh DD, Park JK, Ali M, Danovaro-Holliday MC, Son ND, Hoa NT, Holmgren J, Clemens JD (2006) Long-term effectiveness against cholera of oral killed whole-cell vaccine produced in Vietnam. Vaccine 24(20):4297–4303

Holst J, Feiring B, Naess LM (2005) The concept of "tailor-made", protein-based, outer membrane vesicle vaccines against meningococcal disease. Vaccine 23:2202–2205

Holst J, Martin D, Arnold R, Huergo CC, Oster P, O'Hallahan J, Rosenqvist E (2009) Properties and clinical performance of vaccines containing OMV from *Neisseria meningitidis*. Vaccine 27 (Suppl 2):B3–B12

Howitz M, Grove Krause T, Brunbjerg Simonsen J, Hoffmann S, Frisch M, Munk Nielsen N, Robbins J, Schneerson R, Molbak K, Miller M (2007) Lack of association between group B meningococcal disease and autoimmune disease. Clin Infect Dis 45:1327–1334

Hu L, Joshi SB, Andra KK, Thakkar SV, Volkin DB, Bann JG, Middaugh CR (2012) Comparison of the structural stability and dynamic properties of recombinant anthrax protective antigen and its 2-fluorohistidine-labeled analogue. J Pharm Sci 101(11):4118–4128

Isbrucker R, Arciniega J, McFarland R et al (2014) Report on the international workshop on alternatives to the murine histamine sensitization test (HIST) for acellular pertussis vaccines: state of the science and the path forward. Biologicals 42:114–122

Ivins B, Fellows P, Pitt L, Estep J, Farchaus J, Friedlander A, Gibbs P (1995) Experimental anthrax vaccines: efficacy of adjuvants combined with protective antigen against an aerosol *Bacillus anthracis* spore challenge in guinea pigs. Vaccine 13(18):1779–1784

Ivins BE, Pitt ML, Fellows PF, Farchaus JW, Benner GE, Waag DM, Little SF, Anderson GW Jr, Gibbs PH, Friedlander AM (1998) Comparative efficacy of experimental anthrax vaccine candidates against inhalation anthrax in rhesus macaques. Vaccine 16(11–12):1141–1148

Jackson SG, Goodbrand RB, Ahmed R, Kasatiya S (1995) *Bacillus cereus* and *Bacillus thuringiensis* isolated in a gastroenteritis outbreak investigation. Lett Appl Microbiol 21 (2):103–105

Jendrek S, Little SF, Hem S, Mitra G, Giardina S (2003) Evaluation of the compatibility of a second generation recombinant anthrax vaccine with aluminum-containing adjuvants. Vaccine 21:3011–3018

Jensen SE, Engelhart Illigen KE, Badsberg JH, Haslov KR (2012) Specificity and detection limit of a dermal temperature histamine sensitization test for absence of residual pertussis toxin in vaccines. Vaccine 40:36–40

Jertborn M, Svennerholm AM, Holmgren J (1992) Safety and immunogenicity of an oral recombinant cholera B subunit-whole cell vaccine in Swedish volunteers. Vaccine 10 (2):130–132

Jertborn M, Svennerholm AM, Holmgren J (1993) Evaluation of different immunization schedules for oral cholera B subunit-whole cell vaccine in Swedish volunteers. Vaccine 11:1007–1012

Joellenbeck LM, Zwanziger L, Durch JS, Strom BL (eds) (2002) The anthrax vaccine: is it safe? Does it work? National Academy Press, Washington DC

Kataoka M, Toyoizumi H, Yamamoto A, Ochiai M, Horiuchi Y (2002) CHO cell clustering does not correlate with in vivo histamine-sensitization when measuring residual activity of aldehyde-treated PT. Biologicals 30:297–302

Kaur M, Bhatnagar R (2011) Recent progress in the development of anthrax vaccines. Recent Pat Biotechnol 5(3):148–159

Keller JE (2011) Overview of currently approved serological methods with a focus on diphtheria and tetanus toxoid potency testing. Procedia Vaccinol 5:192–199

Khatami A, Pollard J (2010) The epidemiology of meningococcal disease and the impact of vaccines. Expert Rev Vaccine 9(3):285–298

Kirn TJ, Taylor RK (2005) TcpF is a soluble colonization factor and protective antigen secreted by El Tor and classical O1 and O139 Vibrio cholerae serogroups. Infect Immun 73(8):4461–4470

Kirn TJ, Jude BA, Taylor RK (2005) A colonization factor links Vibrio cholerae environmental survival and human infection. Nature 438(7069):863–866

Knezevic I, Baca-Estrada M, Xing DK, Lei D (2008) WHO working group meeting on standardization of acellular pertussis vaccines: potency assay. Vaccine 26:3960–3968

Laird MW, Zukauskas D, Johnson K, Sampey GC, Henrik Olsen H, Garcia A, Karwoski JD, Cooksey BA, Choi GH, Askins J, Tsai A, Pierre J, Gwinn W (2004) Production and purification of *Bacillus anthracis* protective antigen from *Escherichia coli*. Protein Expr Purif 38:145–152

Lang J, Wood SC (1999) Development of orphan vaccines: an industry perspective. Emerg Infect Dis 5(6):749–756

Larocque RC, Krastins B, Harris JB, Lebrun LM, Parker KC, Chase M, Ryan ET, Qadri F, Sarracino D, Calderwood SB (2008) Proteomic analysis of *Vibrio cholera* in human stool. Infect Immun 76(9):4145–4151

Leppla SH, Robbins JB, Schneerson R, Shiloach J (2002) Development of an improved vaccine for anthrax. J Clin Invest 109:141–144

Levine MM, Lagos R (2004) Vaccination in historical perspective. In: Levine MM (eds) New generation vaccines, 4th edn. Marcel Dekker, New York

Liljeqvist S, Ståhl S (1999a) Production of recombinant subunit vaccines: protein immunogens, live delivery systems and nucleic acid vaccines. J Biotechnol 73:1–33

Liljeqvist S, Ståhl S (1999b) Production of recombinant subunit vaccines: protein immunogens, live delivery systems and nucleic acid vaccines. J Biotechnol 73:1–33

Little SF, Ivins BE, Fellows PF, Pitt MLM, Norris SLW, Andrews GP (2004a) Defining a serological correlate of protection in rabbits for a recombinant anthrax vaccine. Vaccine 22:422–430

Little SF, Webster WM, Ivins BE, Fellows PF, Norris SL, Andrews GP (2004b) Development of an in vitro-based potency assay for anthrax vaccine. Vaccine 22(21–22):2843–2852

Little SF, Ivins BE, Webster WM, Fellows PF, Pitt ML, Norris SL, Andrews GP (2006) Duration of protection of rabbits after vaccination with *Bacillus anthracis* recombinant protective antigen vaccine. Vaccine 24(14):2530–2536

Little SF, Ivins BE, Webster WM, Norris SL, Andrews GP (2007) Effect of aluminum hydroxide adjuvant and formaldehyde in the formulation of rPA anthrax vaccine. Vaccine 25 (15):2771–2777

Liu S, Zhang Y, Moayeri M, Liu J, Crown D, Fattah RJ, Wein AN, Yu ZX, Finkel T, Leppla SH (2013) Key tissue targets responsible for anthrax-toxin-induced lethality. Nature 501 (7465):63–68

Lopez AL, Clemens JD, Deen J, Jodar L (2008) Cholera vaccines for the developing world. Hum Vaccin 4(2):165–169

Lowe CR, Pearson JC (1984) Affinity chromatography on immobilized dyes. In: Jakoby WB (ed) Methods in enzymology 104:97–113

Lyngby J, Olsen LH, Eidem T, Lundanes E, Jantzen E (2002) Quantification of lipopolysaccharide in outer membrane vesicle vaccines against meningococcal disease. High-performance liquid chromatography determination of the constituent 3-hydroxy-lauric acid. Biologicals 30:7–13

Mahalanabis D, Lopez AL, Sur D, Deen J, Manna B, Kanungo S, von Seidlein L, Carbis R, Han SH, Shin SH, Attridge S, Rao R, Holmgren J, Clemens J, Bhattacharya SK (2008) A randomized, placebo controlled trial of the bivalent killed, whole-cell, oral cholera vaccine in adults and children in a cholera endemic area in Kolkata, India. PLoS One 3(6):e2323. doi:10.1371/journal.pone.0002323

Matheny J, Mair M, Mulcahy A, Smith BT (2007) Incentives for biodefense countermeasure development *Biosecurity and Bioterrorism*, vol 5, no 3. © Mary Ann Liebert, Inc

May JC, Progar JJ, Chin R (1984) The aluminum content of biological products containing aluminum adjuvants: determination by atomic absorption spectrometry. J Biol Stand 12:175–183

McDonald C, Inohara N, Nuñez G (2005) Peptidoglycan signaling in innate immunity and inflammatory disease. J Biol Chem 280:20177–20180

Merritt EA, Sarfaty S, Akker FVD, L'Hoir C, Martial JA, Hol WG (1994) Crystal structure of cholera toxin B-pentamer bound to receptor GMl pentasaccharide. Protein Sci 3:166–175

Merritt EA, Kuhn P, Sarfaty S, Erbe JL, Holmes RK, Hol WG (1998) The 1.25 Å resolution refinement of the cholera toxin b-pentamer: evidence of peptide backbone strain at the receptor-binding site. J Mol Biol 282:1043–1059

Metz B, Jiskoot W, Hennink WE, Crommelin DJA, Kersten GFA (2003) Physicochemical and immunochemical techniques predict the quality of diphtheria toxoid vaccines. Vaccines 22:156–167

Miller J, McBride BW, Manchee RJ, Moore P, Baillie LW (1998) Production and purification of recombinant protective antigen and protective efficacy against *Bacillus anthracis*. Lett Appl Microbiol 26(1):56–60

Mosley WH, Ahmad S, Benenson AS, Ahmed A (1968) The relationship of vibriocidal antibody titre to susceptibility to cholera in family contacts of cholera patients. Bull World Health Organ 38(5):777–785

Mosley WH, Aziz KM, Rahman AS, Chowdhury AK, Ahmed A (1973) Field trials of monovalent Ogawa and Inaba cholera vaccines in rural Bangladesh: three years of observation. Bull World Health Organ 49(4):381–387

Nash T (1953) The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochemical 55:416–421

Newman MJ, Powell MF (1995) Immunological and formulation design considerations for subunit vaccines. In: Newman MJ, Powell MF (ed) Vaccine design: the subunit and adjuvant approach. Plenum Press, New York

NIH (1947) Minimum requirements: diphtheria toxoid, 4th edn. US Department of Health, Education Welfare, Bethesda

NIH (1952) Minimum requirements: tetanus toxoid, 4th edn. US Department of Health, Education Welfare, Bethesda

NIH (1953) Minimum requirements: tetanus and diphtheria toxoids combined precipitated adsorbed (for adult use). US Department of Health, Education Welfare, Bethesda

Ochiai M, Yamamoto A, Kataoka M, Toyoizumi H, Arakawa Y, Horiuchi Y (2007) Highly sensitive histamine-sensitization test for residual activity of pertussis toxin in acellular pertussis vaccine. Biologicals 35:259–264

Orenstein WA, Paulson JA, Brady MT, Cooper LZ, Seib K (2013) Global vaccination recommendations and thimerosal. Pediatrics 131:149–150

Pappenheimer AM Jr (1984) Diphtheria. In: Germanier R (ed) Bacterial vaccines. Academic Press Inc, New York

Parreiras PM, Sirota LA, Wagner LD, Menzies SL, Arciniega JL (2009) Comparability of ELISA and toxin neutralization to measure immunogenicity of protective antigen in mice, as part of a potency test for anthrax vaccines. Vaccine 27(33):4537–4542

Pavliak V, Brisson JR, Michon F, Jennings HJ (1993) Structure of the sialylated L3 lipopolysaccharide of *Neisseria meningitidis*. J Biol Chem 268:14146–14152

Peachman KK, Li Q, Matyas GR, Shivachandra SB, Lovchik J, Lyons RC, Alving CR, Rao VB, Rao M (2012) Anthrax vaccine antigen-adjuvant formulations completely protect New Zealand white rabbits against challenge with *Bacillus anthracis* Ames strain spores. Clin Vaccine Immunol 19(1):11–16

Peltola H, Siitonen A, Kataja MJ, Kyronseppa H, Simula I, Mattila L, Oksanen P, Cadoz M (1991) Prevention of travellers' diarrhoea by oral B-subunit/whole-cell cholera vaccine. Lancet 338:1285–1289

Pierce NF, Kaper JB, Mekalanos JJ, Cray WC Jr (1985) Role of cholera toxin in enteric colonization by Vibrio cholerae O1 in rabbits. Infect Immun 50(3):813–816

Pitt ML, Little SF, Ivins BE, Fellows P, Barth J, Hewetson J, Gibbs P, Dertzbaugh M, Friedlander AM (2001) In vitro correlate of immunity in a rabbit model of inhalational anthrax. Vaccine 19:4768–4773

Plotkin SA, Orenstein WA, Offit PA (2008) Vaccines, 5th edn. Licensed Vaccines, Section 2, pp 111–126

Poolman J, OHallander H (2007) Acellular pertussis vaccines and the role of pertactin and fimbriae. Expert Rev Vaccines 6:47–56

Powell BS, Enama JT, Ribot WJ, Webster W, Little S, Hoover T, Adamovicz JJ, Andrews GP (2007) Multiple asparagine deamidation of *Bacillus anthracis* protective antigen causes charge isoforms whose complexity correlates with reduced biological activity. Proteins Struct Funct Bioinf 68:458–479

Rahman MM, Kolli VS, Kahler CM, Shih G, Stephens DS, Carlson RW (2000) The membrane phospholipids of *Neisseria meningitidis* and *Neisseria gonorrhoeae* as characterized by fast atom bombardment mass spectrometry. Microbiology 146:1901–1911

Ramamurthy T, Garg S, Sharma R, Bhattacharya SK, Nair GB, Shimada T, Takeda T, Karasawa T, Kurazano H, Pal A, Takeda Y (1993) Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and Eastern India. Lancet 341(8846):703–704

Ramirez DM, Leppla SH, Schneerson R, Shiloach J (2002) Production, recovery and immunogenicity of the protective antigen from a recombinant strain of *Bacillus anthracis*. J Ind Microbiol Biotechnol 28(4):232–238

Rappuoli R (2007) Bridging the knowledge gaps in vaccine design. Nat Biotechnol 25 (12):1361–1366

Rhie GE, Park YM, Han JS, Yu JY, Seong WK, Oh HB (2005) Efficacy of non-toxic deletion mutants of protective antigen from *Bacillus anthracis*. FEMS Immunol Med Microbiol 45 (2):341–347

Ribot WJ, Powell BS, Ivins BE, Little SF, Johnson WM, Hoover TA, Norris SL, Adamovicz JJ, Friedlander AM, Andrews GP (2006) Comparative vaccine efficacy of different isoforms of recombinant protective antigen against *Bacillus anthracis* spore challenge in rabbits. Vaccine 24:3469–3476

Rinaudo CD, Telford JL, Rappuoli R, Seib KL (2009) Vaccinology in the genome era. J Clin Invest 119:2515–2525

Sack DA, Sack RB, Nair GB, Siddique AK (2004) Cholera. Lancet 363:223–233

Sanchez J, Holmgren J (1989) Recombinant system for overexpression of cholera toxin B subunit in *Vibrio cholerae* as a basis for vaccine development. Proc Natl Acad Sci USA 86:481–485

Sanchez J, Johansson S, Löwenadler B, Svennerholm AM, Holmgren J (1990) Recombinant cholera toxin B subunit and gene fusion proteins for oral vaccination. Res Microbiol 141 (7–8):971–979

Scerpella EG, Sanchez JL, Mathewson III JJ, Torres-Cordero JV, Sadoff JC, Svennerholm AM, DuPont HL, Taylor DN, Ericsson CD (1995) Safety Immunogenicity, and protective efficacy of the whole-cell/recombinant B subunit (WC/rBS) oral cholera vaccine against travelers' diarrhea. J Travel Med 2:22–27

Sekura RD (1988) Novel method of preparing toxoid by oxidation and metal ions. US Patent 4762710

Sekura RD, Fish F, Manclark CR, Meade B, Zhang Y-L (1983) Pertussis toxin: affinity purification of a new ADP-Ribosyl transferase. J Biol Chem 258:14647–14651

Sengupta DK, Sengupta TK, Ghose AC (1992) Antibodies to outer membrane proteins of *Vibrio cholerae* induce protection by inhibition of intestinal colonization of vibrios. FEMS Microbiol Immunol 4(5):261–266

Serruto D, Bottomley MJ, Ram S, Giuliani MM, Rappuoli R (2012) The new multicomponent vaccine against meningococcal serogroup B, 4CMenB: immunological, functional and structural characterization of the antigens. Vaccine 30S:B87–B97

Shin S, Desai SN, Shah BK, Clemens JD (2011) Oral vaccines against cholera. Clin Infect Dis 52 (11):1343–1349

Shrivastaw KP, Singh S (1995a) A new method for spectrophotometric determination of formaldehyde in biologicals. Biologicals 23:47–53

Shrivastaw KP, Singh S (1995b) A new method for spectrophotometric determination of thimerosal in biologicals. Biologicals 23:65–69

Skibinski DA, Baudner BC, Singh M, O'Hagan DT (2011) Combination vaccines. J Glob Infect Dis 3(1):63–72

Soliakov A, Kelly IF, Lakey JH, Watkinson A (2012) Anthrax sub-unit vaccine: the structural consequences of binding rPA83 to Alhydrogel®. Eur J Pharm Biopharm 80(1):25–32

Spangler BD (1992) Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. Microbiol Rev 56(4):622–647

Sperandio V, Girón JA, Silveira WD, Kaper JB (1995) The OmpU outer membrane protein, a potential adherence factor of Vibrio cholerae. Infect Immun 63(11):4433–4438

Stein DM, Robbins J, Miller MA, Lin FY, Schneerson R (2006) Are antibodies to the capsular polysaccharide of *Neisseria meningitidis* group B and *Escherichia coli* K1 associated with immunopathology? Vaccine 24:221–228

Stickings P, Rigsby P, Coombes L, Malik K, Matejtschuk P, Sesardic D (2010) Collaborative study for the calibration of a replacement international standard for diphtheria toxoid adsorbed. Biologicals 38:529–538

Stickings P, Rigsby P, Coombes L, Hockley J, Tierney R, Sesardic D (2011) Animal refinement and reduction: alternative approaches for potency testing of diphtheria and tetanus vaccines. Procedia Vaccinol 5:200–212

Tamura M, Nogimori K, Murai S, Yajima M, Ito K, Katada T, Ui M, Ishii S (1982) Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. Biochemistry 21:5516–5522

Tani C, Stella M, Donnarumma D, Biagini M, Parente P, Vadi A, Magagnoli C, Costantino P, Rigat F, Norais N (2014) Quantification by LC–MSE of outer membrane vesicle proteins of the Bexsero® vaccine. Vaccine 32:1273–1279

Taylor RK, Miller VL, Furlong DB, Mekalanos JJ (1987) Use of phoA gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc Nat Acad Sci USA 84 (9):2833–2837

Tayot J-L, Holmgren J, Svennerholm L, Lindblad M, Tardy M (1981) Eur J Biochem 113:249–258

Tierney R, Stickings P, Hockley J, Rigsby P, Iwaki M, Sesardic D (2011) Collaborative study for the calibration of a replacement international standard for tetanus toxoid adsorbed. Biologicals 39:404–416

Trach DD, Clemens JD, Ke NT, Thuy HT, Son ND, Canh DG, Hang PV, Rao MR (1997) Field trial of a locally produced, killed, oral cholera vaccine in Vietnam. Lancet 349(9047):231–235

Tsai CM, Frasch CE, Rivera E, Hochstein HD (1989) Measurements of LPS (endotoxin) in meningococcal protein and polysaccharide preparations for vaccine usage. J Biol Stand 17:249–258

Tummala M, Hu P, Lee S-M, Robinson A, Chess E (2008) Characterization of pertussis toxin by LC-MS/MS. Anal Biochem 374:16–24

Tummala M, Lee S-M, Chess E, Hu P (2010) Characterization of pertussis toxoid by two-dimensional liquid chromatography-tandem mass spectrometry. Anal Biochem 401:295–302

Tummala M, Chacon A, Chess E, Lee S-M, Hu P (2013) Pertussis toxoid structure: a collaboration and comparison of 2D-LC-MS/MS, UPLC-MS^{E}, and CapLC-MALDI-MS/MS. Anal Biochem 437:40–42

Turnbull PCB (1991) Anthrax vaccines: past present and future. Vaccine 9:533–539

Unnikrishnan M, Rappuoli R, Serruto D (2012) Recombinant bacterial vaccines. Curr Opin Immunol 24:337–342

Use of Anthrax Vaccine in the United States (2000) Recommendations of the advisory committee on immunization practices (ACIP). MMWR Morb Mort Wkly Rep 49(RR-15):1–17

Verma A, McNichol B, Domínguez-Castillo R, Amador-Molina JC, Arciniega JL, Reiter K, Meade BD, Ngundi MM, Stibitz S, Burns DL (2013) Use of site-directed mutagenesis to model the effects of spontaneous deamidation on the immunogenicity of *Bacillus anthracis* protective antigen. Infect Immun 81(1):278–284

NIH Source. http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid=8325Taxonomy. *Vibrio cholera*, proteins: 5, chemicals: 6 modified: 2011/09/10 00:00, MMDB ID: 8325 PDB ID: 3CHB

Vipond C, Suker J, Jones C, Tang C, Feavers I, Wheeler J (2006) Proteomic analysis of a meningococcal outer membrane vesicle vaccine prepared from the group B strain NZ98/254. Proteomics 6:3400–3413

Vollmer W, Blanot D, De Pedro M (2008) Peptidoglycan structure and architecture. FEMS Microbiol Rev 32:149–167
Wagner L, Verma A, Meade BD, Reiter K, Narum DL, Brady RA, Little SF, Burns DL (2012) Structural and immunological analysis of anthrax recombinant protective antigen adsorbed to aluminum hydroxide adjuvant. Clin Vaccine Immunol 19(9):1465–1473
Waldor MK, Mekalanos JJ (1994) *Vibrio cholera* O139 specific gene sequences. Lancet 343 (8909):1366
Wassil J, McIntosh E, Serruto D, DeTora L, Bröker M, Kimura A (2012) Clin Invest 2(5):503–517
Watkinson A, Soliakov A, Ganesan A, Hirst K, Lebutt C, Fleetwood K, Fusco PC, Fuerst TR, Lakey JH (2013) Increasing the potency of an alhydrogel formulated vaccine by minimising antigen–adjuvant interactions. Clin Vaccine Immunol 20(11):1659–1668
Weil AA, Arifuzzaman M, Bhuiyan TR, LaRocque RC, Harris AM, Kendall EA, Hossain A, Tarique AA, Sheikh A, Chowdhury F, Khan Al, Murshed F, Parker KC, Banerjee KK, Ryan ET, Harris JB, Qadri F, Calderwood SB (2009) Memory T cell responses to *Vibrio cholera* O1 infection. Infect Immun 77:5090–5096
WHO (1965) BLG/UNDP 77.1
WHO (1990) Requirements for diphtheria, tetanus, pertussis and combined vaccines. World Health Organ Tech Rep Ser 800:87–151
WHO (1992) Annex 1, Good manufacturing practices for biological products (WHO Tech Rep Series No. 822)
WHO (1998) Annex 2, guidelines for the production and control of the acellular pertussis component of monovalent or combined vaccines. WHO Tech Rep Ser 878:57–70
WHO (2009) Cholera: global surveillance summary, 2008. Wkly Epidemiol Rec 84:309–324
WHO (2013a) Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines. WHO Expert committtee on biological standaradization. Sixty-second report, Geneva, WHO Technical report series, No 979, Annex 4, pp 189–235
WHO (2013b) Manual for quality control of diphtheria, tetanus and pertussis vaccines
WHO (2014a) Recommendations to assure the quality, safety and efficacy of tetanus vaccines (adsorbed). WHO Expert committtee on biological standaradization. Sixty-third report, Geneva, WHO technical report series, No 980, Annex 5, pp 271–333
WHO (2014b) Recommendations to assure the quality, safety and efficacy of diphtheria vaccines (adsorbed). WHO expert committtee on biological standaradization. Sixty-third report, Geneva, WHO technical report series, No 980, Annex 4, pp 211–270
Williamson ED, Hodgson I, Walker NJ, Topping AW, Duchars MG, Mott JM, Estep J, Lebutt C, Flick-Smith HC, Jones HE, Li H, Quinn CP (2005) Immunogenicity of recombinant protective antigen and efficacy against aerosol challenge with anthrax. Infect Immun 73(9):5978–5987
Wirz M, Gentilli G, Collotti C (1990) Tetanus vaccine: present status. In: Mizrahi A (ed) Bacterial vaccines, Alan R. Liss, Inc. New York
Wistreich GA, Anthrax: a bioterroist weapon. Copyright © 2001 By RC Educational Consulting Services, Inc
Wolfe DN, Florence W, Bryant P (2013) Current biodefense vaccine programs and challenges. Hum Vaccine Immunotherapeutics 9(7):1591–1597
World Health Organization (1991) WHO report on cholera: ancient scourge on the rise. WHO announces global plan for cholera control. WHO Feature (154):1–3
World Health Organization (2000) WHO report on global surveillance of epidemic-prone infectious disease, pp 39–54. http://www.who.int/csr/resources/publications/surveillance/WHO_report_Infectious_Disease.Pdf. Accessed 2013
World Health Organization (2004) Guidelines for the production and control of inactivated oral cholera vaccines. WHO technical report series 924, Annex 3, Geneva. http://www.who.int/biologicals/publications/trs/areas/vaccines/cholera/en/index.html
World Health Organization (2010) Cholera vaccines: WHO position paper. Wkly Epidemiol Rec 85:117–128

World Health Organization Strategic Advisory Group of Experts (SAGE) on Immunization (2009) Background paper on the integration of oral cholera vaccines into global cholera programs, pp 1–74. http://www.Who.int/immunization/sage/1-Background_Paper__Cholera_Vaccine_FinalDraft_13_oct_v2.pdf

Xing D, Gaines-Das R, Newland P, Corbel M (2002) Comparison of the bioactivity of reference preparations for assaying *Bordetella pertussis* toxin activity in vaccines by the histamine sensitization and Chinese hamster ovary-cell tests: assessment of validity of expression of activity in terms of protein concentration. Vaccine 20:3535–3642

Xing D, Maes A, Behr-Gross M-E, Costanzo A, Daas A, Buchheit KH (2010) Collaborative study for the standardization of the histamine sensitizing test in mice and the CHO cell-based assay for the residual toxicity testing of acellular pertussis vaccines. Pharmeur Bio Sci Notes 1:51–63

Xing D, Yuen C-T, Asokanathan C, Rigsby P, Horiuchi Y (2012) Evaluation of an in vitro assay system as a potential alternative to current histamine sensitization test for acellular pertussis vaccines. Vaccine 40:456–465

Yuen C-T, Canthaboo C, Menzies JA, Cyr T, Whitehouse LW, Jones C, Corbel MJ, Xing D (2002) Detection of residual pertussis toxin in vaccines using a modified ribosylation assay. Vaccine 21:44–52

Yuen C-T, Horiuchi Y, Asokanathan C, Cook S, Douglas-Bardsley A, Ochiai M, Corbel M, Xing D (2010) An in vitro assay system as a potential replacement for the histamine sensitization test for acellular pertussis based combination vaccines. Vaccine 28:3714–3721

Zomber G, Reuveny S, Garti N, Shafferman A, Elhanany E (2005) Effects of spontaneous deamidation on the cytotoxic activity of the *Bacillus anthracis* protective antigen. J Biol Chem 280(48):39897–39906

Chapter 7
Bacterial Polysaccharide Vaccines: Analytical Perspectives

Earl Zablackis, Philippe Talaga and Suddham Singh

7.1 Introduction

The development of conjugate vaccines has improved public health and lowered incidence of bacterial disease due to their ability to induce memory as well as impart herd immunity in vaccinated populations. However, global disease burden still remains high (WHO 2012); therefore, there is still a need for earlier developed polysaccharide vaccines. Table 7.1 highlights the rationale for continued production and use of polysaccharide vaccines.

The capsular polysaccharides of pathogenic bacteria are extracellular polysaccharides that exist on the surface of both gram negative and gram positive bacteria (e.g., *Neisseria meningitidis* and *Streptococcus pneumoniae*, respectively). The capsule functions include protection against dehydration and modulating the flow of nutrients to the bacterial cell surface and aid the bacterium in establishing infection. Bacterial species for which polysaccharide vaccines exist are divided into serogroups (or serotypes) that are based on their structurally and immunologically distinct capsular polysaccharides. Different bacterial species have different numbers of known serotypes. There are more than 90 serotypes of *Streptococcus pneumoniae*, 6 *Haemophilus influenzae* serotypes and at least 12 *Neisseria meningitidis* serotypes. Different serotypes (or serogroups) of the same organism may have different infectivity's. Polysaccharide capsules are immunogenic, however, being T

E. Zablackis (✉)
Manufacturing Technology, Sanofi Pasteur, Swiftwater, USA
e-mail: Earl.Zablackis@sanofipasteur.com

P. Talaga
R&D, Sanofi Pasteur, Marcy l'Étoile, France
e-mail: Philippe.Talaga@sanofipasteur.com

S. Singh
Vaccine Research & Early Development, Pfizer Inc., Pearl River, NY, USA
e-mail: Suddham.Singh@pfizer.com

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6_7

Table 7.1 Polysaccharide vaccine disease burden (adapted from (Jones 2005) see references therein)

Organism	Disease	Death rate or morbidity
Streptococcus pneumoniae	Acute respiratory infections and meningitis	>1 million deaths (plus many cases neurological damage)
Neisseria meningitidis	Meningitis and bacteremia	500,000 cases/50,000 deaths (plus many cases neurological damage)
Salmonella enterica serovar typhi	Typhoid	21.6 million cases, more than 200,000 deaths

Table 7.2 Principal licensed bacterial polysaccharide vaccines

Trade name	Manufacturer	Valency	Bacterial species and strain
Menomune-A/C/Y/W-135	Sanofi Pasteur, Inc.	4	*Neisseria meningitidis*: serotypes A, C, Y, W-135
Pneumo 23	Sanofi Pasteur Inc.	23	*Streptococcus pneumoniae*: serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9 N, 9 V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 20, 22F, 23F, 33F
Pneumovax 23	Merck & Co, Inc.		
Pnu-Immune 23	GlaxoSmithKline		
Typhim Vi	Sanofi Pasteur, Inc.	1	*Salmonella enterica* serovar typhi

cell-independent Type II antigens keeps them from eliciting immunologic memory, and therefore polysaccharide vaccines require repeated immunizations. In addition, they are poorly immunogenic in infants, thus the development of polysaccharide-protein conjugate vaccines to protect infants, the group most at risk for these bacterial infections. However, the polysaccharide vaccines remain viable vaccines due to their use with adolescents and adults. Due to the presence of multiple serotypes, different infectivity of different serotypes, differential geographical distributions of serotypes, and differential age group prevalence, many polysaccharide vaccines contain multiple polysaccharide antigens.

There are currently five major vaccines manufactured and licensed against bacterial pathogens that consist of purified bacterial capsular polysaccharides (Table 7.2). The vaccines contain from 1 to 23 specific serotype polysaccharides.

The following sections discuss the composition, structure, and the analytical methodologies used to test and release polysaccharides for vaccine use.

7.2 Polysaccharide Production

7.2.1 Isolation

Bacterial capsular polysaccharides are isolated from fermentation cultures. The isolation and purification processes will vary depending on the bacterial phenotype

(gram class), location of polysaccharide (excreted or membrane bound), and composition of polysaccharide (backbone sugars, linkage types, side groups, charge type and charge distribution). Isolation will vary depending on how well the polysaccharides adhere to the cell membrane, either the wet cell mass is recovered by centrifugation and the polysaccharides extracted or in the case of secreted polysaccharides, the polysaccharides are recovered by various filtration methods. Chemical agents (e.g., Cetavalon,) are often used to assist in isolation of the polysaccharides depending on the nature of the composition of the particular polysaccharide.

7.2.2 Purification

Purification as with isolation can be achieved with a variety of methods depending on the nature of the polysaccharide to be purified. Typical processes include combinations of selective precipitation, diafiltration, and/or chromatographic steps.

7.2.3 Testing

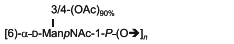
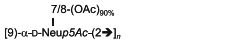
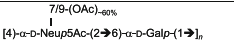
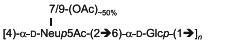
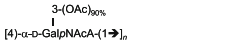
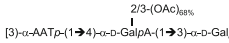
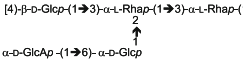
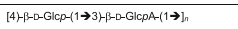
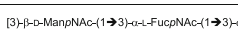
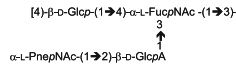
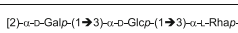
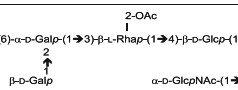
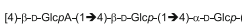
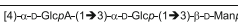
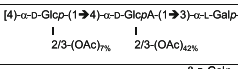
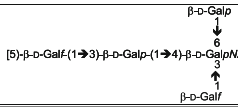
Testing of polysaccharides encompasses identification, composition, purity, impurities, size, and stability. Polysaccharides used for vaccines are tested to meet criteria established by the World Health Organization (WHO), Pharmacopoeia, and individual product licenses with various National Health Authorities. The following sections describe the test methodologies adapted for vaccine polysaccharides.

7.3 Polysaccharide Composition and Structure

7.3.1 Composition: Repeat Unit

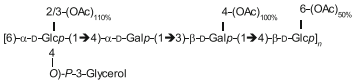
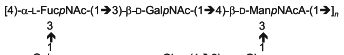
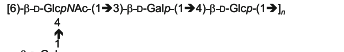
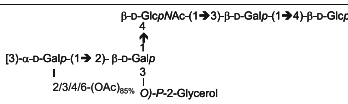
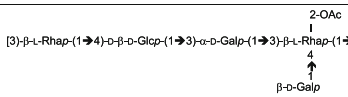
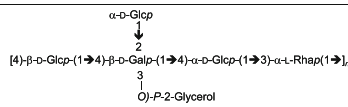
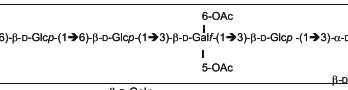
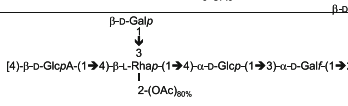
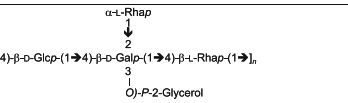
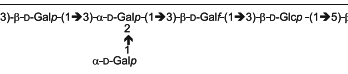
The bacterial capsular polysaccharides are high molecular weight polymers (ranging typically from less than 50,000 to more than 1,000,000 Daltons (WHO 1966)) consisting of a distinct repeat unit that may be either a single monosaccharide or an oligosaccharide consisting of two or more monosaccharides. These repeat units may be branched or linear, typically linked through glycosidic bonds but in some cases linked through a phosphodiester bond. In addition, they contain a variety of substituted side groups, e.g., *O*-acetyl, pyruvate, and phosphoglycerol. Table 7.3 lists the characteristic repeat units for each of the polysaccharides used in licensed vaccines today.

Table 7.3 Licensed bacterial polysaccharide vaccines

Serotype*	Primary structure	References
Men A		Lemercinier and Jones (1996)
Men C		Lemercinier and Jones (1996)
Men W-135		Lemercinier and Jones (1996)
Men Y		Lemercinier and Jones (1996)
Vi		Heyns and Kiessling (1967)
PnPS 1		Stroop et al. (2002)
PnPS 2		Jansson et al. (1988)
PnPS 3		Reeves and Goebel (1941)
PnPS 4		Jones et al. (1991)
PnPS 5		Jansson et al. (1985)
PnPS 6B		Kenne et al. (1979)
PnPS 7F		Moreau et al. (1988)
PnPS 8		Jones and Perry (1957)
PnPS 9N		Rutherford et al. (1994)
PnPS 9V		Rutherford et al. (1991)
PnPS 10A		Jones (1995)

(continued)

Table 7.3 (continued)

Serotype*	Primary structure	References
PnPS 11A		Calix et al. (2011 and 2012a)
PnPS 12F		Leontein et al. (1981)
PnPS 14		Lindberg et al. (1977)
PnPS 15B		Jones and Lemercinier (2005)
PnPS 17F		Beynon et al. (1997), Jones et al. (2000)
PnPS 18C		Lindberg (1990), Kamerling and Tomasz (2000), Chang et al. (2012)
PnPS 19A	[4)-β-D-ManpNAc-(1➔4)-α-D-Glcp-(1➔3)-α-L-Rhap-1-*P*-(O➔]$_n$	Katzenellenbogen and Jennings (1983)
PnPS 19F	[4)-β-D-ManpNAc-(1➔4)-α-D-Glcp-(1➔2)-α-L-Rhap-1-*P*-(O➔]$_n$	Jennings et al. (1980)
PnPS 20		Beynon et al. (1997)
PnPS 22F		Richards et al. (1989)
PnPS 23F		Richards and Perry (1988)
PnPS 33F		Richards et al. (1984), Lemercinier and Jones (2006)

***Men:** *Neisseria meningitidis;* PnPs: *Streptococcus pneumoniae*; Vi: *Salmonella enterica* Serovar Typhi

7.3.2 Colorimetric Methods: Polysaccharide Quantitation

Traditionally, polysaccharide content (on a dry weight basis) has been estimated using a range of colorimetric assays specific to a class of monosaccharide (e.g., hexose, hexosamine, uronic acid, or methyl pentose) or to functional groups (e.g., phosphodiester) that are specific to the polysaccharide antigen. Due to fact that assays were developed prior to most structures being known and the reference standard for such methods is typically a pure monosaccharide or similar model compound, component quantification may not parallel true stoichiometry. Thus, manufacturers have developed correlations among alternative approaches. Purity can be calculated based on the method employed, the salt form present, idealized repeat unit mass, average side chain component mass, and % moisture/solvents. Table 7.4 lists colorimetric tests that may be appropriate to determine the composition of polysaccharides, the analyte, and the polysaccharide to which the method is appropriate. Attention must be paid to the response factors for the sugar units in the polysaccharide as they may differ from those of a pure monosaccharide reference standard. Manufacturers address these concerns during method validation.

For simple polysaccharide content measurements where identity is not important, e.g., in-process monitoring of polysaccharide yield, it has been common practice to use a generic colorimetric method specific for a type of monosaccharide (e.g., Anthrone method for hexose) that is present in each of the polysaccharides, for all serotypes manufactured as this lowers the number of tests needed for process monitoring.

7.3.2.1 Uronic Acids

The Carbazole method of Gregory (1960) is used to quantitate uronic acid content in pneumococcal polysaccharide serotypes 1, 2, 3, 5, 8, 9 N, 9 V, and 22 F. The polysaccharide is heated with a sulfuric acid/borate solution to release the monosaccharides, the sample is then cooled and a solution of carbazole/ethanol added and allowed to react for 15 min and then absorbance read at 530 nm. Uronic acid content is quantitated against a standard curve prepared with a glucuronic acid standard.

7.3.2.2 Hexosamines

Hexosamine content is determined using a modified Morgan-Elson assay (Kao and Tsai 2004). Polysaccharide ($\sim$5 mg) is digested with HCl, cooled, and then reacted with a solution of acetylacetone/sodium carbonate and then heated again, cooled, and reacted with Ehrlich's reagent (dimethylamino benzaldehyde in alcohol), and absorbance read at 530 nm. Hexosamine content is quantitated against a standard curve prepared with reference standard glucosamine. A modification of this method

Table 7.4 Colorimetric methods used for polysaccharide quantification

PS antigen	Orcinol assay (Bial reagent)	Molybdate (Chen) assay	Resorcinol assay (Seliwanoff reagent)	Sulfuric acid digestion	Carbazole assay	Dimethyl-amino- benz-aldehyde assay	Cysteine sulfuric acid assay	Hestrin assay[a]	Anthrone-Sulfuric acid assay	Resorcinol-Sulfuric acid assay
	Ribose	Phosphate	Sialic acid	Total nitrogen	Uronic acids	Hexosamines	Methyl-pentoses	*O*-acetyl	Total sugar	Total sugar
Hib PRP	X	X								Y
Men-A		X				X		X		
Men-C			X					X		
Men-Y			X			X		X	X	X
MenW135			X			X		X	X	X
Pneumo (serotype specific)		X	X	X	X	X	X	X	X	X
Vi					X			X		Y

X = Assay considered appropriate for use with these polysaccharides, Y = Assay considered appropriate for estimating in-process chromatography fractions for process development

[a] The Hestrin assay is appropriate for compositional analysis and identity, but because the degree of *O*-acetylation can vary between polysaccharide batches it is not normally a suitable assay for polysaccharide quantification

for quantitating total hexosamine and differentiating galactosamine without interference from glucosamine is available as well (Blumenkrantz and Asboe-Hansen 1976).

7.3.2.3 Sialic Acid

Sialic acid content is typically determined using the colorimetric method of Svennerholm (1957). Polysaccharide is hydrolyzed with resorcinol reagent (resorcinol/HCl/$CuSO_4$) by heating on a boiling water bath. Absorbance is read at 580 nm. A purified N acetyl neuraminic acid standard is used for the calibration curve. An additional method by Warren, commonly referred to as the thiobarbituric assay, is also available (Warren and Odin 1959).

7.3.2.4 Methyl Pentose

Methyl pentose (6 deoxy-hexose) content is determined using the method of Kabat (1967). Polysaccharide is dissolved in water and hydrolyzed with sulfuric acid. Samples are allowed to react with cysteine hydrochloride and then the absorbance read at 396 nm and again at 430 nm. The absorbance difference (to account for hexose interference) is used to calculate the content against a standard curve prepared with rhamnose reference standard.

7.3.2.5 Total Carbohydrate Via Hexose

The total carbohydrate content of polysaccharides can be determined by a general test for Hexose content, for example the anthrone method, which has recently been updated to an automated microtiter plate format (Turula et al. 2010). Sugars react with the anthrone reagent under acidic conditions to yield a blue-green color. Polysaccharide is dissolved in water and hydrolyzed with sulfuric acid containing the anthrone reagent. The solution is then allowed to cool and its absorbance is measured at 620 nm. There is a linear relationship between the absorbance and the amount of sugar that was present in the original sample. This method determines both reducing and non-reducing sugars because of the presence of the strongly oxidizing sulfuric acid. As with other methods it is nonstoichiometric and therefore it is necessary to prepare a calibration curve using a series of standards of known carbohydrate concentration. For general use, Glucose is used as standard.

An alternative anthrone assay has been developed (Rondel et al. 2013) that takes measurements at two wavelengths (560 and 620 nm) in order to adjust for responses of both neutral hexoses and uronic acids, thus allowing for simultaneous quantitation of both types of sugars in a single assay.

7.3.3 Colorimetric Methods: Specific Sugar Substituent's

7.3.3.1 *O*-Acetyl

The method of Hestrin (1949) is used to quantitate the number of *O*-acetyl substitutions in each polysaccharide. Polysaccharide are dissolved in water and hydrolyzed with hydrochloric acid in presences of hydroxylamine and ferric chloride. Upon completion of reaction the absorbance is read at 540 nm. The amount of *O*-acetyl is determined against a standard curve of acetylcholine reference standard.

7.3.3.2 Pyruvate

Pyruvate content can be determined using the diphenylhydrazine method (Slonecker and Orentas 1962; Friedemann and Haugen 1943; Anthon and Barrett 2003). Polysaccharide dissolved in water is hydrolyzed with HCl in presence of diphenylhydrazine. Absorbance is read at 515 nm. Caution should be used when using the diphenylhydrazine method since other α-keto acids present can interfere. An alternative method, the definitive lactate dehydrogenase method of Hadjivassiliou and Reider (1968) can also be used. Pyruvic acid is cleaved from the polysaccharide by acid hydrolysis with 0.4 N oxalic acid. Hydrolysate is neutralized with $CaCO_3$ and the supernatant is diluted with water, 1 M triethanolamine, and a solution of NADH added. Absorbance is read at 340 nm and then lactate dehydrogenase added and absorbance read at 1 min intervals until constant. The percentage pyruvate is then determined by calculation based on weight, initial and final absorbance values, and correction factors.

Note: most analysis today is performed directly by NMR.

7.3.3.3 Phosphate (PO_4)

Phosphorous content of polysaccharides can be determined using one of several methods e.g., the method of Chen et al. (1956) or Bartlett (1959). In the Chen method, polysaccharide is dissolve in water and then hydrolyzed in the presence of sulfuric acid and perchloric acid. Hydrolysate is reacted with ammonium molybdate reagent and ascorbic acid until blue color stabilizes. Absorbance is read at 820 nm and PO_4 is quantitated against a standard of potassium dihydrogen phosphate or NIST traceable Phosphorus standard.

In the method of Bartlett (modification of Fiske and SubbaRow method (Fiske and SubbaRow 1925)), phosphate esters are released from the polysaccharide by acid hydrolysis (sulfuric acid; H_2O_2 added mid reaction) to yield inorganic phosphate, which is converted to ammonium phosphomolybdate upon the addition of ammonium molybdate. Ammonium phosphomolybdate is quantitatively reduced to

a blue molybdenum complex by 1-amino-2-naphthyl-4-sulfonic acid in the presence of sulfite and bisulfite, the Fiske-SubbaRow reagent. Absorbance is read at 830 nm and PO_4 is quantitated against a standard of potassium dihydrogen phosphate or NIST traceable P standard.

7.3.4 Chromatographic Methods: Monosaccharide Composition

There are a variety of chromatographic methods (HPLC, HPAEC, and GC) and capillary electrophoresis (CE) methods available today for monosaccharide composition and quantitation of polysaccharides. The methods depend on specific hydrolysis conditions to preserve specific structural aspects of the component monosaccharides. Hydrolysis is then followed by separation of the monosaccharides. Hydrolysis is optimized to release monosaccharides with as little modification to the monosaccharide as possible. Hydrolysis may be followed by derivatization (e.g., attachment of fluorophore tag) prior to chromatographic separation. Separation can be performed using various technologies, for example by HPLC, HPAEC-PAD/PED (Lei et al. 2000), GC, GC-MS (Kim et al. 2005), or CE (Lamb et al. 2005).

7.3.4.1 HPLC

7.3.4.2 Fluorescent Labeling

Several different fluorescent molecules are available for tagging monosaccharides (2-Aminobenzoic acid [2-AA], 2-Aminobenzamide [2-AB], 2-Aminopyridine [2-AP], and Trisodium 8-aminopyrene-1,3,6-trisulfonic salt [APTS]) by attachment via reductive amination and then detecting with a fluorescent detector. Separation can be done via normal phase, reverse phase, or anion exchange HPLC or CE. Polysaccharide is hydrolyzed to monosaccharide components and then the fluorescent label is added by reductive amination to the released monosaccharides. The labeled monosaccharides may be cleaned up prior to HPLC or CE analyses.

Monosaccharide composition of pneumococcal polysaccharides has been accomplished using a fluorescent detection method (Canaan-Haden et al. 2006). Polysaccharides are subjected to sequential hydrolysis (48 % HF followed by 2 M TFA). The released monosaccharides are then re-N-acetylated and labeled with 2-AB by reductive amination in DMSO/CH_3COOH. For quantitation of polysaccharides, a monosaccharide reference mixture (Gal, Glc, Rha, ManNAc, GlcNAc, and GalNAc) is labeled and used for quantitation of polysaccharides. Labeled sugars are then separated using rp-HPLC with a 7.8 × 300 mm C18 TSK ODS120T column and detected using a Fluorescence detector.

7.3.4.3 HPAEC-PAD

A major improvement in polysaccharide analysis has been the introduction of high-performance anion exchange chromatography (HPAEC) coupled to pulsed amperometric detection (PAD). This powerful analytical technique permits detection of picomole amounts of sugars with minimal sample clean up, and is amenable to automation using a computer-controlled autosampler. This technique has become one of the mainstays of analysis for monosaccharide composition analysis as no derivatization is required. It is essentially the only method for simultaneous identification and quantitation of unlabeled monosaccharides.

Due to the fact that carbohydrates are weak acids with pKa values in the range of 12–14, and at high pH they become ionized, they can be separated as oxyanions, on nonporous polymeric stationary phases with MicroBeads having quaternary ammonium functional groups, by ion-exchange chromatography. Pulsed Amperometric Detection is used since it relies on high pH solutions that HPAEC utilizes. In the amperometric detection system (electrochemical cell), various potentials are applied over a specific period of time and a current is generated from the oxidation or reduction of monosaccharide at the electrode surface. A typical amperometric detection system contains a working electrode (gold) and a reference electrode. The cyclic pulse between positive and negative potentials applied to the electrode maintains a stable active electrode surface by limiting oxidation products from fouling the electrode over time. The current generated by the oxyanions is proportional to the monosaccharide concentration, thus this system allows for direct quantitation of monosaccharides without derivatization.

Typical polysaccharide composition analysis is performed by first hydrolyzing the polysaccharide to release the monosaccharide components. Hydrolysis conditions are optimized to the polysaccharide of interest to preserve the structural integrity of the particular monosaccharides in the different polysaccharides. TFA is the most commonly used acid; however, HCl or HF may be used depending on the nature of the monosaccharides and their linkage within the polysaccharide being analyzed.

The method(s) used for the hydrolysis of glycosidic linkages can be considered to be the major limitation of accurate carbohydrate analysis and quantification of polysaccharides by HPAEC-PAD, as hydrolysis of a complex polysaccharide to produce a quantitative yield of all the constituent monosaccharides can be difficult.

Acid or base hydrolysis depolymerizes polysaccharides into oligosaccharides, monosaccharides, or smaller fragments that are polysaccharide specific for the optimized hydrolysis conditions employed. These hydrolysis products can be quantified directly by use of HPAEC-PAD or HPAEC-CD (conductivity detection) for ions. Caution should be used during development of the hydrolysis conditions due to the fact that aggressive hydrolysis conditions can destroy some components of the polysaccharide in many cases. Typical HPAEC-PAD chromatograms for three pneumococcal serotypes are presented in Fig. 7.1.

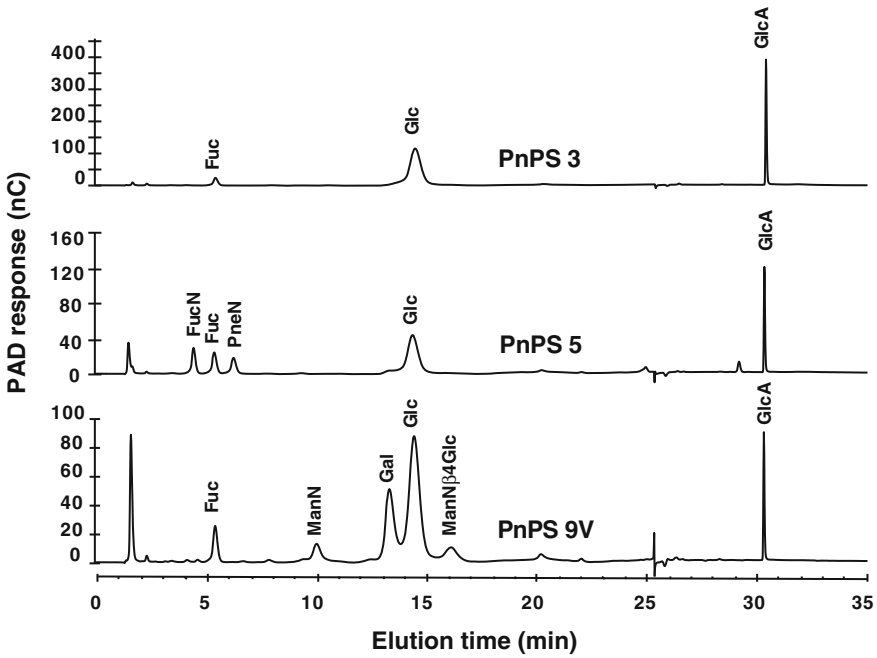


Fig. 7.1 HPAEC–PAD chromatograms of polysaccharide hydrolysates. Each of the polysaccharides (1 μg injected) was hydrolyzed using methanolysis followed by TFA hydrolysis. Adapted from Talaga et al. (2002), reprinted with permission from Elsevier Limited

Haemophilus Influenzae Type B Polysaccharide

Although there are no polysaccharide-only vaccines containing Hib polysaccharide, the analysis of Hib polysaccharide is mentioned here because Tsai et al. (1994) first developed an HPAEC-PAD method to quantitate the polysaccharide of *Haemophilus Influenzae* type b (Hib) and the methodology is currently used to quantitate the unconjugated polysaccharide in Hib conjugate vaccines. The Hib capsular polysaccharide is a polymer consisting of the repeating unit -3)-β-D-Rib*f*-(1)-β-D-ribitol-5-*P*-(O-, also termed polyribosylribitol phosphate or PRP. PRP is depolymerized in NaOH solution to yield ribitol-ribose-phosphate and the quantitation against a purified PRP is performed after HPAE chromatography using a Dionex CarboPac™ PA1 column.

Several adaptations and modifications of this method are available for quantifying both total and unconjugated PRP in conjugated Hib and combination vaccines (Lei et al. 2000; Haan et al. 2013). Bardotti et al. (2000) described an alternative method for the quantification of PRP based on TFA hydrolysis that resulted in the quantitative release of ribitol and ribose. The quantitation was against ribitol after HPAE chromatography using a Dionex CarboPAc™ MA1 column.

Meningococcal Polysaccharides

Ricci et al. (2001) described an HPAEC-PAD method for Meningococcal type A polysaccharide based on quantitation of the Mannosamine-6-phosphate released by hydrolysis with TFA. Bardotti et al. (2008) described the quantitation of MenW135 and Meningococcal type Y also by hydrolysis with TFA followed by HPAEC using a CarboPAc™ PA1 column. Galactose and glucose were used as analytical and quantitation standards. Meningococcal type C polysaccharide has been determined by HPAEC-PAD after hydrolysis with HCl or TFA, as described by Vipond et al. (2012).

Lei et al. (2000) described a procedure for quantification of free polysaccharides in tetravalent meningococcal conjugate vaccine after separation from the conjugates via selective DOC precipitation followed by TFA hydrolysis and then HPAEC using a CarboPac™ PA10.

Pneumococcal Polysaccharides

Talaga et al. (2002) investigated three different hydrolysis procedures for analysis of pneumococcal polysaccharides: (1) TFA hydrolysis; (2) methanolysis followed by TFA hydrolysis; (3) HF hydrolysis followed by TFA hydrolysis. Chromatography was performed on a CarboPac™ PA10. TFA hydrolysis was found to be satisfactory for most of the serotypes based on the identification of quantitative recovery of at least one monosaccharide to enable quantification. Methanolysis followed by TFA hydrolysis was most appropriate for serotypes that contained uronic acids and HF followed by TFA was most appropriate for serotypes containing phosphate residues in their repeating units.

Salmonella Enterica Serovar Typhi Vi Polysaccharides

For the Vi polysaccharide, Micoli et al. (2011) described a strong alkaline hydrolysis (2 N NaOH, 4 h at 110 °C) resulting in a single peak by HPAE chromatography on CarboPac™ PA1 column. The Vi polysaccharide was quantitated against a purified Vi polysaccharide hydrolyzed in parallel.

Polysaccharide Substituents

HPAEC with CD has also been used for compositional analysis of bacterial polysaccharides. Kao and Tsai (2004) developed procedures for the determination of *O*-acetyl, *N*-acetyl, pyruvate, and phosphate residues in meningococcal, pneumococcal, and Vi polysaccharides. For *O*–Ac determination, polysaccharide was hydrolyzed with NaOH (10 mM @ 37 °C) and separated on either Dionex AS11-HC or AS15 columns. The HPAEC method showed good correlation to the Hestrin

method (Hestrin 1949) although results were slightly higher using HPAEC. For phosphate determination, polysaccharide was hydrolyzed with HF (38 %) and separated on an AS11-HC column. Pyruvate content was analyzed by selective hydrolysis (10 mM HCl @ 100 °C for 20 min) of the pyruvic ketal on the Galactose residue of pneumococcal type 4 polysaccharide. The pyruvate was then separated using HPAEC with an AS11-HC column coupled with conductivity detector.

HPAEC methods for polysaccharide quantitation should be developed with adequate controls to maintain the suitability of the methods. Response factors for each monosaccharide should be calculated for absolute quantitation of monosaccharides in order to more accurately estimate the polysaccharide content. Equally important is the use of a suitable reference polysaccharide. Adequate controls for the hydrolysis and any cleanup steps prior to HPAEC analysis should be built into the method. Methods should also contain appropriate system suitability to protect against issues such as detector drift, electrode/conductivity cell fouling etc. A typical method should have one or more control samples that can be used to monitor the hydrolysis step, the cleanup step, and the chromatography step.

7.3.4.4 Gas Chromatography (GC)

Polysaccharide characterization is a complex and challenging task due to the inherent diversity and chain irregularities exhibited by bacterial polysaccharides. Gas chromatography coupled with mass spectrometry (GC-MS) has been used as a quantitative tool for polysaccharides analysis for decades. The technique is used to determine the monosaccharide constituents, linkage position between the monosaccharide residues, position of the monosaccharide in the chain (terminal/reducing end, branched/intra chain), and the ring size (five or six member) (Walford 2010; Merkle and Poppe 1994). Excellent resolution and robustness are typically associated with GC applications due to the large number of theoretical chromatographic plates and the inherent purity of the final derivatized sample solubilized into organic solvent. However, polysaccharide samples must undergo a multistep sample treatment that involves depolymerization into constituent monosaccharides and then derivatization. Typical detectors used for monosaccharide analysis include the flame ionization detector (FID) and the mass selective detector (MSD). The latter provides additional confirmation of peak assignments by analysis of mass spectra as well as peak purity.

For GC analysis, two general approaches are typically used. The first approach employs hydrolysis followed by reduction and peracetylation and then GC separation and detection either by FID (where retention time of sample is compared to authentic monosaccharide standard) or MS with chemical ionization using ammonia to confirm mass of the alditol acetates. The second approach employs permethylation followed by hydrolysis, reduction, and peracetylation then separation by GC and MS detection. Various hydrolysis and derivatization techniques are used depending on the nature of the monosaccharides present in the polysaccharide.

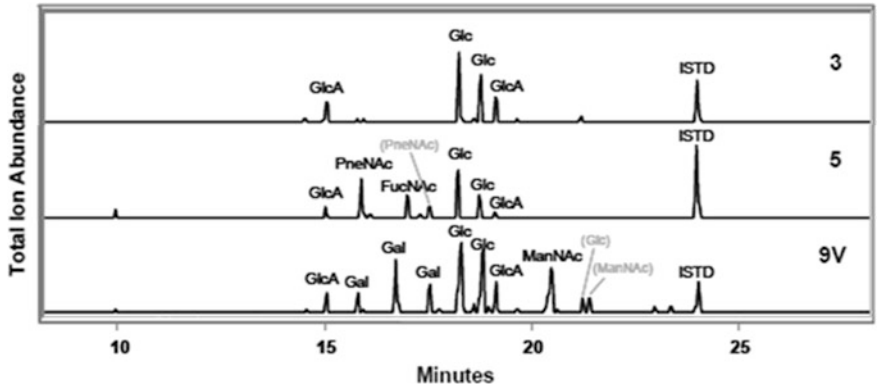


Fig. 7.2 GC–MSD total ion chromatograms for the three pneumococcal polysaccharides. The amounts of polysaccharide tested ranged from 0.1 to 0.5 mg, with 20 μg of myoinositol included as an internal standard (ISTD). Adapted from Kim et al. (2005) reprinted with permission from Elsevier Limited

Kim et al. (2005) developed a simple GC analysis method for Pneumococcal polysaccharides and conjugates. Polysaccharide solutions containing a myoinositol internal standard were depolymerized to monosaccharide constituents by methanolysis (methanolic 3 N HCl @ 121 °C for 2 h). Samples were re-N-acetylated (methanol/pyridine/acetic anhydride 30 min) following methanolysis then derivatized with TMS to form TMS methyl glycosides (Trisil reagent @ 80 °C for 20 min). Samples were then analyzed by GC-MSD equipped with a capillary column and separated over a ramped temperature gradient.

Quantitation of polysaccharide is then based on total recovered monosaccharide relative to authentic monosaccharide standards (except Pn-1 where Pn-1 std is used). Response factors for each monosaccharide are calculated and applied for quantitation of each sample. The mass spectra of the TMS methylglycosides yield identity and peak purity information as well. Results from GC-MS analysis were comparable to wet chemical colorimetric methods (92–123 %). Example Total Ion Chromatograms for three pneumococcal serotypes are presented in Fig. 7.2.

7.3.4.5 Capillary Electrophoresis (CE)

Capillary Electrophoresis has become a useful tool for polysaccharide as well as monosaccharide quantitative analysis. Capillary Zonal Electrophoresis (CZE) has been shown to be a robust technique for the separation and identification of a mixture of group A, C, Y, and W-135 polysaccharides (Lamb et al. 2005); additionally quantitation was achieved without the need for colorimetric assays or composition analysis.

Free solution capillary zone electrophoresis (CZE) with simple phosphate/borate separation buffers is used to separate intact, native polysaccharides of meningococcal

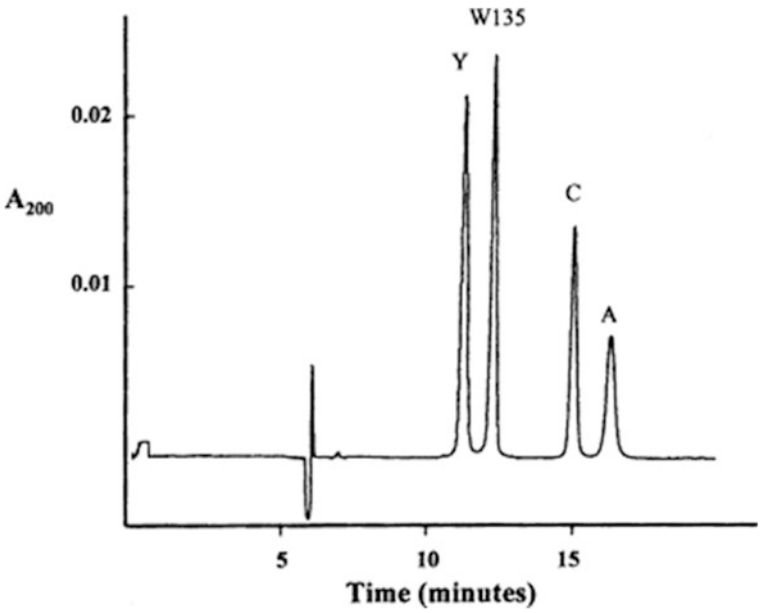


Fig. 7.3 Separation of a mixture of four meningococcal native polysaccharides using 40 mM phosphate/40 mM borate (natural pH) in a bare silica capillary, 37 cm (30 cm effective length) × 50 μm (I.D.) Separation was conducted using 10 kV (normal polarity) at 20 °C. Serogroup identity of peaks as marked. Adapted from Lamb et al. (2005), reprinted with permission from Elsevier Limited

serogroups A, C, Y, and W-135 (Fig. 7.3). Separation is robust with respect to variations in test conditions and behaves as expected with respect to changes in temperature, ionic strength, and addition of an organic modifier. Separation of serogroups W-135 and Y (repeat unit composed of sialic acid residues alternating with either galactose or glucose, respectively) is achieved using phosphate buffer and is therefore not dependent on differential complexation with borate. Addition of sodium dodecyl sulfate to the separation buffer (i.e., MEKC) resulted in peak splitting for all four serogroups. Changes in polysaccharide size did not affect migration time for the size range examined, but serogroup C polysaccharide (a sialic acid homopolymer) was separable from sialic acid monosaccharide. CZE quantification of multiple lots of each of the four serogroups was compared to wet chemical determination by phosphorus or sialic acid measurement. Results from CZE determination showed good agreement with the wet chemical methods. CZE recoveries compared to the wet chemistry results were 98–103 % for serogroup A, 91–100 % for serogroup C, 97–101 % for serogroup W, and 97–102 % for serogroup Y (with one exception at 127 %).

7.3.5 Nephelometry

Nephelometry can also be used as either an in-process test for yield determinations or as a bulk polysaccharide quantitation method. Both rate and end point Nephelometry methods can be used. The methodology relies on the formation of antigen–antibody aggregates and detection of these by light scattering. Antiserum is mixed with antigen and scattered light is measured. The end point nephelometry method allows the antibody–antigen reaction to go to completion until all possible complexes are formed. In rate nephelometry the rate of light scatter is measured as the reagents are added. Reagent is kept constant so the rate of change is directly related to the amount of antigen in solution. Optimal concentrations of both antigen and antibody are needed to avoid inhibition of the precipitin reaction (reduction of antigen–antibody complex) due to excess amounts of antigen and/or antibody. Antibodies specific to each polysaccharide to be quantitated are needed when using nephelometry for quantitation of mixtures of polysaccharides.

Vaccine polysaccharides can be quantitated by rate nephelometry (Lee 1983, 1984). Rate nephelometry has been applied to both multivalent pneumococcal polysaccharide (14 and 23 valent) vaccines and meningococcal polysaccharide vaccines (4 valent) (Lee 1983). Purified polysaccharide of each type is used as standards. Specific antisera for each polysaccharide are produced and purified (compounds such as fibrinogen or lipids in crude sera can interfere with the light scattering). A polyvalent standard curve containing each polysaccharide in the vaccine formulation is used to quantitate the polysaccharides. The method can produce results within 20 % of target on an individual polysaccharide basis and within 3 % of target on an overall basis. Nephelometry can also be used as an alternative immunologic technique for polysaccharide identification testing.

7.4 Polysaccharide Identity Tests

The identity of individual bulk polysaccharides used in vaccine manufacture is required. In cases where other polysaccharides are produced on the same manufacturing site, the method should be validated to show that it distinguishes the desired polysaccharide from all other polysaccharides produced on that manufacturing site. The polysaccharide identity has traditionally been determined by a combination of several methods, including immunologic techniques (*WHO, EP vaccine monographs*), wet chemical methods for characteristic carbohydrate and others components (Table 7.4), and more recently, NMR spectroscopy.

7.4.1 Immunologic Methods

Immunologic methods require access to highly specific antisera that are able to distinguish between closely related polysaccharide antigens. Commonly used

formats for this purpose are immunoprecipitation, immunoelectrophoresis, and enzyme-linked immunosorbent methods (ELISA). The (serological) specificity of the antiserum should be demonstrated by the absence of cross reactivity with heterologous polysaccharides manufactured at the same site. The selective nature of the tests means that although they may confirm the presence of the antigen tested for, they give no information regarding other material that may be present.

7.4.1.1 Dot Blot

One of the simplest immunologic methods to be applied to polysaccharide identity testing is the Dot Blot method. It is a non-electrophoretic method where polysaccharide (antigen) is applied to a membrane, unbound sites are then blocked, a specific/selective primary antibody added followed by secondary antibody which is conjugated to an enzyme such as horseradish peroxidase followed by addition of suitable substrates which results in a colored precipitate. Mixtures of polysaccharides can also be analyzed for the identity of the multiple components with this methodology if one has selective non-cross-reactive primary antibodies to each of the polysaccharides in the mixture.

7.4.1.2 ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) methodology is commonly used for polysaccharide quantitation due to the availability of highly selective antisera-containing antibodies specific to the polysaccharide to be quantitated. The method uses standard polysaccharides for identification; these should be qualified reference polysaccharides with known concentration and stability. The methods also typically have additional positive controls e.g., in case of test for polysaccharide in a multivalent product, a positive control lot is used for monitoring assay performance independent of the production lots tested. This positive control should also be a qualified test material with known stability and concentration.

The typical method consists of coating a 96-well plate with the polysaccharide antigen, controls, and standards. The plate is allowed to incubate several hours and then washed to remove excess antigen (some methods may incorporate a blocking step after coating and this first wash). Antisera-containing antibodies specific to individual polysaccharides or specific antibody solutions are then added to the plate and allowed to incubate for the required time then washed to remove excess antibody. A secondary antibody that typically has horseradish peroxidase conjugated to it is then added and allowed to incubate for the required time and then washed to remove excess reagents. Finally, enzyme substrate containing a dye is added and color is allowed to develop the required time and the reaction is stopped and absorbance measured. Each sample of a multivalent mixture should appear positive to each of the serospecific antibodies.

Note: Test may be slightly modified to be a quantitative method for content of individual polysaccharides.

7.4.2 NMR

NMR spectroscopy is an extremely powerful analytical technique for determining the composition and structure of polysaccharides. NMR spectra are uniquely capable of revealing all of the structural features of polysaccharides, within one analysis, and are sensitive to subtle structural changes. Also, two clear advantages for performing an NMR analysis on polysaccharides are that NMR is a nondestructive method and no chromophore groups or derivation steps are required for detection.

All chemically different protons have chemically different electronic environments. Proton NMR spectra contain characteristic signals depending on the electronic environment of the protons present and their relative intensities can confirm the proportions of different sugar residue types and substituents. The selectivity of 1D proton NMR permits differentiation between very closely related polysaccharides that differ by only a glycosidic linkage such as Pn 19A/19F (→3)-α-L-Rha$_p$/→2)-α-L-Rha$_p$). Example proton spectra demonstrating the differentiating ability of NMR for structurally closely related polysaccharides are presented in Fig. 7.4. Such differentiation is impossible to achieve with the traditional colorimetric tests.

NMR methods require access either to authentic samples of the polysaccharide or to reference spectra. Comparison is made visually in terms of the positions, relative intensities, and multiplicities of significant resonances or by use of other objective methods. The first approach for NMR identity testing is to compare the fingerprint NMR spectrum of a test sample with that of a reference sample acquired under identical conditions. The reference spectrum may have been acquired previously and stored electronically. For such an approach, all the polysaccharides produced at the manufacturing site should have a distinctive spectrum. Comparison of both spectra can be done either visually or with use of other objective methods such as computer-assisted correlation coefficient calculations for characteristic regions of the test and reference spectra (Abeygunawardana et al. 2000). If the degree and location of *O*-acetyl, affect the appearance of spectra, an in situ de-*O*-acetylation can be performed in order to simplify the spectrum. Quantitation of *O*-acetyl residues can be achieved the same way by comparison of generated *O*-acetate intensities to that of an added internal standard (Jones 2005).

The second approach is to use NMR in a quantitative manner and compare the intensities of characteristic resonances of different sugars expected to be present in the polysaccharide test sample. A ratio of the different sugar classes present can be calculated. Quantitation can be achieved by comparison of these intensities to that of an added internal standard (Abeygunawardana et al. 2000) or by use of electronic reference signal i.e., use of ERETIC method (Akoka et al. 1999).

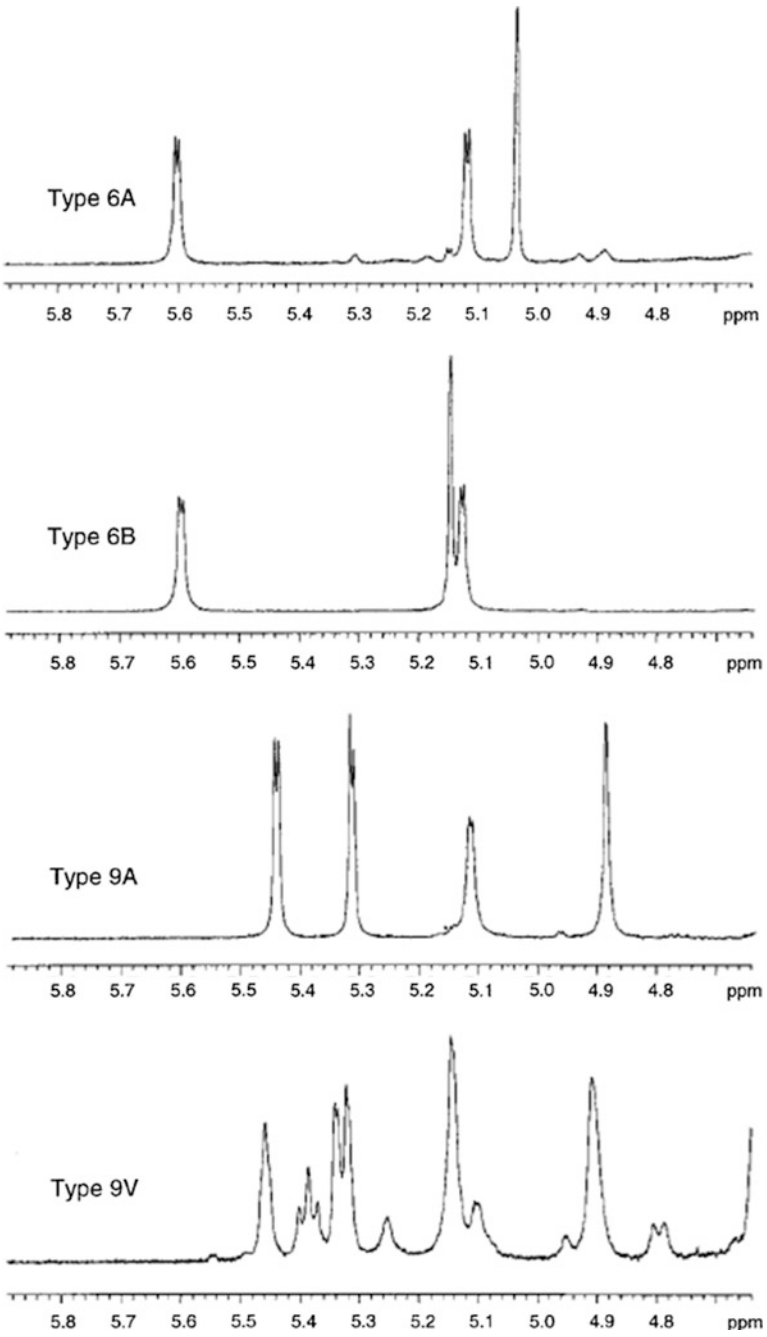


Fig. 7.4 The identity regions of the 600 MHz ^{1}H NMR spectra for *S. pneumoniae* serotypes 6A, 6B, 9A and 9 V. Adapted from Abeygunawardana et al. (2000), reprinted with permission from Elsevier Limited

The ability to characterize molecules at the atomic level has allowed NMR spectroscopy to play a leading role in the structural identification of several new pneumococcal serotypes discovered within serogroups which were considered to be well-characterized by serological methods (Calix et al. 2011, 2012a, b; Oliver et al. 2013; Park et al. 2007; Zartler et al. 2009). NMR data identifying the position of *O*-acetylation of polysaccharide vaccines have been published; this allows determination of the position and extent of *O*-acetylation of polysaccharide lots which may be important for antigenicity and for the subsequent activation reaction if based upon hydroxyl groups. For polysaccharides that contain variable *O*-acetylation resulting in spectral complexity that hampers the use of NMR as a polysaccharide identity test, a second spectrum can be recorded after simplifying the spectrum by performing base-catalyzed de-*O*-acetylation in the NMR tube. This approach has been reported for meningococcal polysaccharides (Jones and Lemercinier 2002), but can be applied to pneumococcal serotypes Pn 1, 7 F, 9 V, and 18 C as well.

For instrumentation and experimental considerations for use of NMR spectroscopy in routine GMP use, see USP < 761 >, or EP 2.2.33.

7.5 Polysaccharide Purity

Purity of polysaccharides has been traditionally determined by application of compendial methods for the levels of impurities present in the polysaccharide bulk dry powders. Residual components from the culture and purification processes, i.e., water, solvents, nucleic acids, and protein that may be present in the polysaccharide powders are quantitated. Other impurities for which there are no compendial test methods may be present e.g., phenol, CTAB, various antifoams, and are analyzed by specific in-house methods by the manufacturer.

7.6 Additional Polysaccharide Characterization

7.6.1 Molecular Size

The molecular size distribution of the purified polysaccharide reflects manufacturing consistency and has been documented as an important parameter for the immunogenicity of polysaccharides preparations. As an indicator of the structural integrity of the polysaccharides in general, this parameter is of utmost importance for polysaccharides that are susceptible to depolymerization (for example meningococcal group A and pneumococcal serotypes 6A, 6B, 19A and 19F polysaccharides). Historically and as stated in WHO and Pharmacopoeia guidelines, the methods are based on conventional gel filtration chromatography using soft gels columns, such as Sepharose. After collection and colorimetric analysis of fractions

or refractive index detection, the results are reported as the distribution coefficient (K_D) determined from the main peak of the elution curve or as the percentage of material eluting before a defined K_D cut-off value. At best, molar mass estimates relative to secondary polymer standards of known molecular weights averages (e.g., dextran or pullulan) can also be calculated.

Development and improvements in the manufacturing process and product quality of highly crosslinked macroporous gels that can be used at higher flow rates and pressures has made development of High-Performance Size Exclusion chromatography (HPSEC) possible. For such columns to work successfully in water, the hydrophilicity of the packing material must be increased, either by secondary treatment of organic gel or by alteration of the synthesis stage using different monomers.

An HPSEC experiment employing only a single concentration detector (RI or UV) can be termed Conventional Gel permeation chromatography as the results are expressed the same way as using soft-gels columns (either by calculating KD or the relative molecular weights average relative to a secondary series of standards). Such methods have been developed for meningococcal polysaccharides group A, C, Y, and W135 (Von-Hunolstein et al. 2003), *Haemophilus influenzae* type b and pneumococcal polysaccharides (Bussat et al. 1990). Forced degradation studies have shown that these methods were sensitive enough to detect molecular size distribution modifications. For example, the elution profile of Men-A PS (Von-Hunolstein et al. 2003), PRP, and pneumococcal serotype 19F (Bussat et al. 1990) changed when stored at 37 °C for different period of time, however, Men-C, Men-Y, and MenW135 were not depolymerized under such conditions.

The disadvantage of these methods is that SEC columns separate on the basis of size not molecular weight, which can lead to discrepancies if the standards used for calibration do not have absolutely the same structures in solution as the samples. Moreover, other physical parameters of capsular polysaccharides (e.g., absolute molecular size and weight distribution, rigidity, drainage, shape, and structure) are very limited.

There is increasing interest in so-called multidetector HPSEC systems, employing one or more detectors that respond to molecular properties other than concentration. Static light Scattering (see Fig. 7.5) and viscometry detectors are the most common detectors in these applications, with the concomitant use of both devices (along with the required concentration detectors) termed “triple detection”.

Static light-scattering detectors respond directly to the molecular weights of the sample and do not rely on a column calibration, and can also give information regarding the size of the polysaccharides (Rg) with the use of Multi-Angle Light-Scattering detector. Viscometry detectors give the intrinsic viscosity of the polysaccharide, a property that can be related to the density of the polysaccharides. Therefore, triple detection can be used to directly define the distribution of molecular size, molar mass, and hydrodynamic volume.

These parameters can in turn be used to define changes in the segment density, rigidity, and structure of the polysaccharides in solution as a function of molecular size and molar mass. For example, calculation of the Mark–Houwink–Sakurada

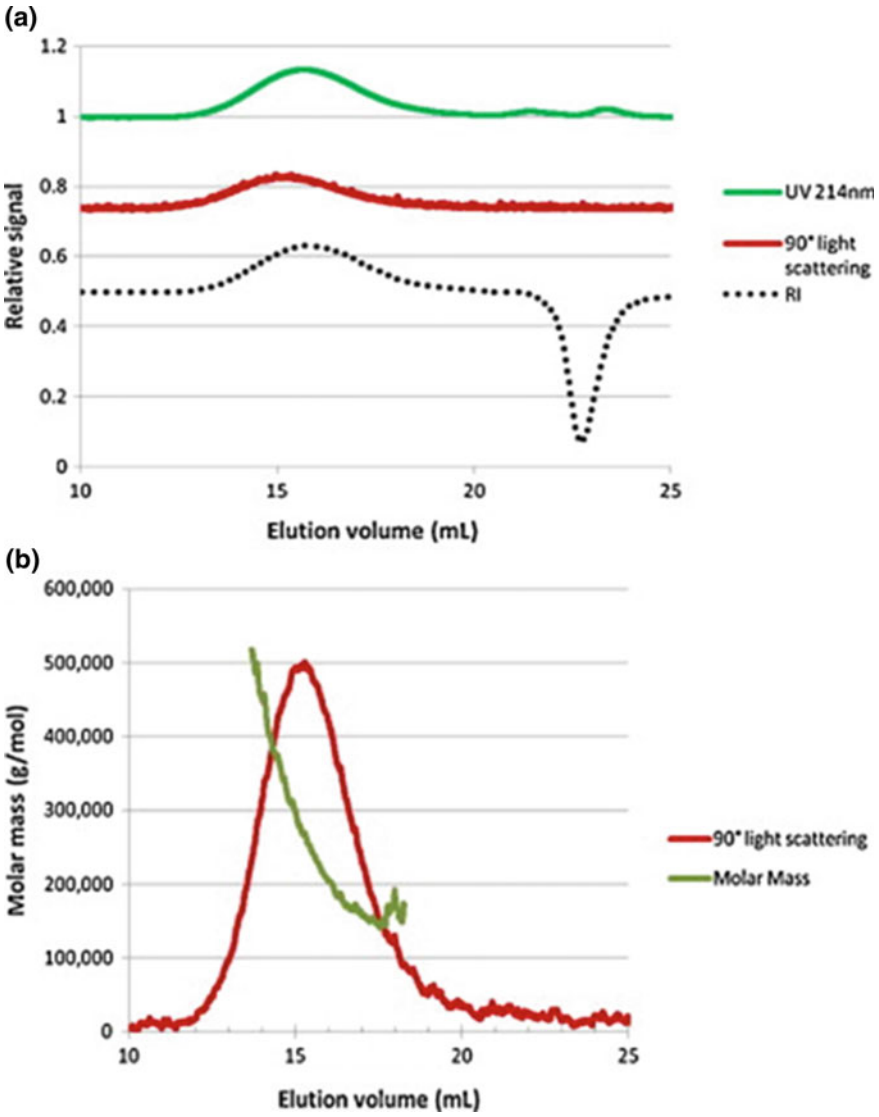


Fig. 7.5 HPLC–SEC elution profile of purified polysaccharide sample 'BF 7/07 A Pooled' as monitored by MALLS superimposed on molar mass versus volume curve. The serogroup X polysaccharide sample was eluted in PBS, pH 7.4 on a TSK G6000 PWXL + G5000 PWXL column. Adapted from Xie et al. (2012), reprinted with permission from Elsevier Limited

coefficients yields information about the structures of polysaccharides in solution (e.g., sphere, random coils, stiff coils or rods like conformations).

One such triple detection method has been developed for pneumococcal polysaccharides (Bednar and Hennessey 1993) and is used as a release test for molecular

size on pneumococcal polysaccharide drug substances. This assay provides a direct measure of the absolute weight-average molecular mass (Mw) of polysaccharide size distributions in units of kilodaltons (kDa). Minimum release specifications (a separate value for each of the 23 serotypes) have to be established on the basis of production experience and these specifications have to be substantially greater than the molecular sizes that exhibited reduced polysaccharide immunogenicity. Replacement of conventional gel permeation chromatography by HPSEC methods is also possible and a correlation between gel permeation chromatography and HPSEC has to be established (Bussat et al. 1990) both on native and forced degraded polysaccharide samples, in order to determine the most relevant HPSEC parameters for setting new specifications based on these parameters (Thiébaud et al. 2014).

Due to the polyvalent nature of polysaccharide vaccine final formulations, an HPSEC triple detection method cannot be used to monitor polysaccharide molecular size in the formulated final product and an alternative approach has to be developed. This can be achieved by adding off-line rate nephelometric detection (HPSEC/RN) to yield relative molecular size of each serotype in a multivalent vaccine. Data from the two molecular size measurements is used to establish a correlation between relative molecular size and absolute weight-average molecular mass (Mw) and for each serotype, this correlation permits the direct alignment of the relative mass molecular size specification in the final formulated product with the Mw specification for the monovalent polysaccharide preparation (Sweeney et al. 2000; MacNair et al. 2005). This procedure has been very useful in characterizing the stability profile of each pneumococcal polysaccharide in the drug product as (i) a significant decrease in relative molecular size was detected for serotype 19A and 19F under normal storage conditions at 5 °C, and for serotype 5, 19A, and 19F and 5 under forced degradation by incubation at 60 °C; and (ii) Serotypes 1, 9 V, and 18 C displayed significant decreases in relative antigenicity over time under normal storage at 5 °C. This loss in size and antigenicity can be attributed to the loss of *O*-acetyl residues. However, the decrease in antigenicity could not be definitively linked to the loss of immunogenicity for these polysaccharides.

Use of HPSEC for MW characterization is limited to the study of the polysaccharide and conjugate preparations only in appropriate mobile phases that permit necessary quantitative chromatographic elution for the analysis. For example, when capsular polysaccharides are lipidated, quantitative elution can be difficult to achieve due to aggregation of capsular polysaccharides and/or hydrophobic interactions with the stationary phase. In such cases, addition of detergents in the mobile phase may be necessary (Thiébaud et al. 2014) but detergents may be incompatible with light-scattering detection. Moreover, quantitative elution of very large aggregates (and crosslinked conjugates) can be very difficult to achieve due to the low exclusion limit of HPSEC with respect to the size of such compounds.

Orthogonal methods to HPSEC such as Analytical Ultracentrifugation (AUC) or Asymmetrical Flow Field Flow fractionation (A4F) can be used to characterize the hydrodynamic properties of polysaccharides and conjugates in very different solutions.

A4F is a family of flexible elution techniques capable of simultaneous separation based on molar size with the same on-line detectors used in HPSEC. The sample domain extends from about 1 nm to 100 μm without stationary phase interactions or large applied forces. This has proven to be complementary to HPSEC for large polymers such as starches, carrageenans (Bourgoin et al. 2008) etc., however, to our knowledge, there has been very limited application to vaccine polysaccharides (or conjugates). The use of on-line Dynamic Light Scattering (DLS) as an additional detector for both HPSEC and A4F can be advantageous. One big advantage of adding a DLS detector is the ability to obtain enhanced resolution as compared with batch mode DLS analysis. Although batch mode is simple and quick, there is the one drawback of poor baseline resolution (3 to 1 in diameter). Moreover, results generated by this technology in batch mode can be difficult to interpret on compounds with both high polydispersity and highly intricate motions, both in amplitude and time scales. Therefore, chromatographic separation can help to obtain more accurate and precise absolute hydrodynamic size (Dh, hydrodynamic diameter without any calibration).

In an analytical ultracentrifuge, a sample being spun is monitored in real time through an optical detection system. Monitoring the sedimentation of macromolecules in the centrifugal field allows for their hydrodynamic and thermodynamic characterization in solution, without any interaction from any matrix or surface. Determination of pneumococcal capsular polysaccharide sedimentation and molar mass distributions (obtained by sedimentation velocity and equilibrium experiments) and the independent method of HPSEC-MALS-VISc were used to show that 10 polysaccharides from all the serotypes studied had semi-flexible structures in solution with persistence lengths in the range from 4 to 9 nm. (Harding et al. 2012)

To obtain even more information on the general shapes and the influence of different counter ions in polysaccharide solutions, Atomic Force Microscopy (AFM) experiments can also be performed (see Fig. 7.6). The main advantage of this technology is that direct images of the macromolecules are generated. For Meningococcal type A polysaccharides, this technique has revealed that ammonium counter ions were associated with the presence of single polymer chains in an elongated conformation, whereas sodium counter ions favored the folding of the chains into a more globular conformation. The addition of calcium ions produced the aggregation of a limited number of globular polysaccharide chains to form a "toroidal-like" structure (Foschiatti et al. 2009).

7.6.2 Other Components

Polysaccharide purification processes are not 100 % efficient at delivering only the polysaccharide of interest. Processing chemicals as well as other biomolecules such as polysaccharides, lipids, and nucleic acids may be present in the purified polysaccharides. NMR analysis can also be used to identify and potentially quantify

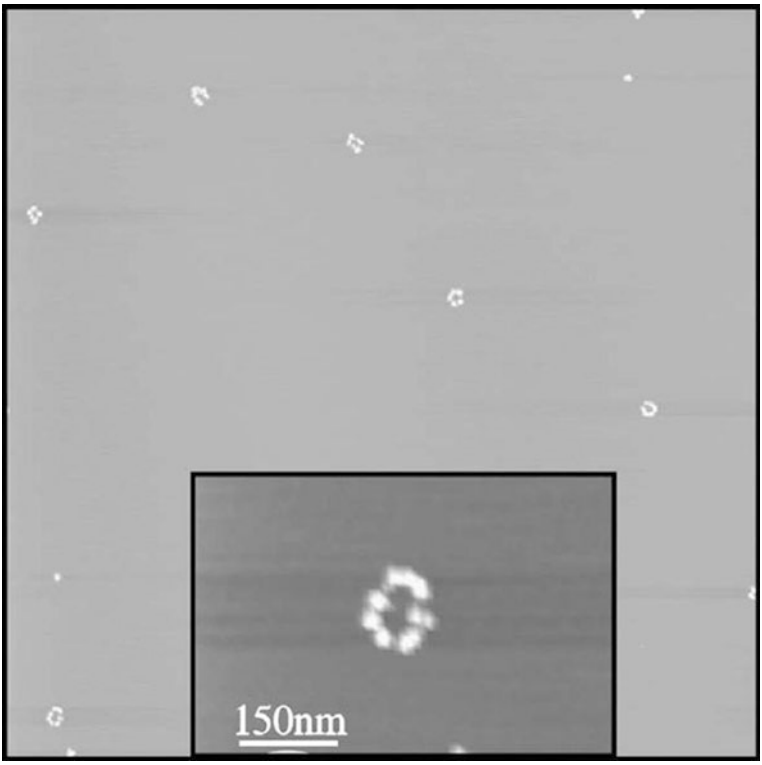


Fig. 7.6 AFM image of the Ca^{2+} salt form of Mn A spray-dried on mica; polymer concentration 5 μg/mL. An enlargement of the object at the bottom left corner is shown in the inset. Adapted from Foschiatti et al. (2009), reprinted with permission from Elsevier Limited

other organic materials present in the polysaccharide preparations such as contaminants, process residuals, and product residuals such as cell wall polysaccharide (CWPS) from pneumococcal polysaccharides (Abeygunawardana et al. 2000). CWPS is composed of a tetrasaccharide repeating unit joined by ribitol phosphodiester bonds with phosphocholine substituents. Quantitation has been reported by use of ^{1}H or ^{31}P NMR analysis, of the phosphocholine moiety (Karisson et al. 1999), or (depending on the serotype), by ribitol analysis with GC-MS and HPAEC-PAD (Talaga et al. 2002). Residual CWPS may be chemically linked to the capsular polysaccharide (bound) or present by itself (free); these have been quantitated by two methods: labeling CWPS with a fluorescent tag followed by SEC-HPLC-RI/FL and by an NMR method based on self-diffusion rates (Xu et al. 2005). The associated peptidoglycan may be quantified by the same techniques.

7.7 Reference Materials (There are Very Few!)

There are relatively few reference materials available for polysaccharides. Certified reference materials are available for monosaccharides e.g., Glc, NANA and P, which can be used with colorimetric assays for polysaccharide quantitation. However, there is almost a complete lack of suitable reference standards for whole bacterial polysaccharides used in vaccine production. Such polysaccharide standards would be ideal for identity testing, quantitation, or molecular size determination. To date, the only polysaccharide standard available is for Men-C polysaccharide is available from NIBSC (Vipond et al. 2012).

7.8 Future Developments

Due to the success of polysaccharide conjugate vaccines with their ability to induce memory response and extended age range, development of new polysaccharide alone vaccines is limited. However, there is still a large demand for such vaccines and therefore there is need for modernization of testing programs with implementation of modern analytical technologies. Just as HPAEC-PAD and NMR have gradually been implemented as replacement analytical methodologies for vaccine polysaccharide analysis, other methodologies are being investigated for use as replacement methodologies. Inductively Coupled Plasma-Atomic emission spectroscopy (ICP-AES) MS is currently being used for P content and polysaccharide quantitation (Swartz et al. 2000). It is certain that as other promising technologies are developed they will certainly be assessed for application to polysaccharide analysis.

References

Abeygunwardana C, Williams TC, Sumner JS, Hennessey JP Jr (2000) Anal Biochem 279:226–240
Akoka S, Barantin L, Trierweiler M (1999) Anal Chem 71:2554–2557
Anthon G, Barrett D (2003) J Sci Food Agric 83:1210–1213
Bardotti A, Averani G, Berti F, Berti S, Carinci V, D'Ascenzi S, Fabbri B, Giannini S, Giannozzi A, Magagnoli C, Proietti D, Norelli F, Rappuoli R, Ricci S, Costantino P (2008) Vaccine 26:2284–2296
Bardotti A, Ravenscroft N, D'Ascenzi S, Averani G (2000) Vaccine 18:1982–1993
Bartlett GR (1959) J Biol Chem 234:466–468
Bednar B, Hennessey JP (1993) Carbohydr Res 243:115–130
Beynon LM, Richards JC, Perry MB (1997) Eur J Biochem 250:163–167
Blumenkrantz N, Asboe-Hansen G (1976) Clin Biochem 9:269–274
Bourgoin A, Zablackis E, Poli JB (2008) Food Hydrocolloids 22:1607–1611
Bussat B, Schultz Arminjon R, Valentin C, Armand J (1990) Biologicals 18:117–121
Calix JJ, Nahm MH, Zartler ER (2011) J Bacteriol 193:5271–5278
Calix JJ, Nahm MN, Zartler ER (2012a) J Bacteriol 194:206

Calix JJ, Porambo RJ, Brady AM, Larson TR, Yother J, Abeygunwardana C, Nahm MH (2012b) J Biol Chem 287:27885–27894
Canaan-Haden L, Cremata J, Chang J, Valeds Y, Cardoso F, Bencomo VV (2006) Vaccine 24S2: S2/70–S2/71
Chang J, Serrano Y, Garrido R, Rodriguez LM, Pedroso J, Cardoso F, ValdesY, Garcia D, Fernandez-Santana V, Verez-Bencomo V (2012) Vaccine 30:7090–7096
Chen PS, Toribana TY, Warner H (1956) Anal Chem 28:1756
Fiske CH, SubbaRow YJ (1925) J Biol Chem 66:375
Foschiatti M, Hearshaw M, Cescutti P, Ravenscroft N, Rizzo R (2009) Carbohydr Res 344:940–943
Friedemann TE, Haugen GE (1943) J Biol Chem 147:415–442
Gregory JD (1960) Arch Biochem Biophys 89:157–159
Haan A, Put RM, Beurret M (2013) Biomed Chromatogr 27:1137–1142
Hadjivassiliouk AG, Rieder SV (1968) Clin Chim Acta 19:357
Harding, Stephen E et al (2012) Carbohydr Polym 90:237–242
Hestrin S (1949) J Biol Chem 180:249–261
Heyns K, Kiessling G (1967) Carbohydr Res 3:340–353
Jansson PE, Lindberg B, Andersson M, Lindquist U, Henrichsen J (1988) Carbohydr Res 182:111–117
Jansson PE, Lindberg B, Lindquist U (1985) Carbohydr Res 140:101–110
Jennings HJ, Rosell K-G, Carlo DJ (1980) Can J Chem 58:1069–1074
Jones C (1995) Carbohydr Res 269:175–181
Jones C, Currie F, Forster MJ (1991) Carbohydr Res 221:9121
Jones C, Lemercinier X (2002) J Pharm Biomed Anal 30:1233–1247
Jones C, Lemercinier X (2005) Carbohydr Res 340:403–409
Jones C, Whitley C, Lemercinier X (2000) Carbohydr Res 325:192–201
Jones CJ (2005) An Acad Bras Cienc 77:293–324
Jones JKN, Perry MB (1957) J Am Chem Soc 79:2787–2793
Kabat EA (1967) Experimental immunochemistry. In: Kabat EA, Mayer M (eds) Academic Press, New York, pp 538–543
Kao G, Tsai C-M (2004) Vaccine 22:335–344
Karisson C, Jansson P-E, SØrensen UBS (1999) Eur J Biochem 265:1091–1097
Kamerling JP, Tomasz A (2000) Pneumococcal polysaccharide: a chemical view–Streptococcus pneumoniae–molecular biology and mechanisms of disease. Mary Ann Lierbert, Inc., Larchmont, pp 81–114
Katzenellenbogen E, Jennings HJ (1983) Carbohydr Res 124:235–245
Kenne L, Lindberg B, Madden JK (1979) Carbohydr Res 73:175–182
Kim JS, Laskowich ER, Arumugham RG, Kaiser RE, MacMichael GJ (2005) Anal Biochem 347:247–272
Lamb DH, Lei QP, Hakim N, Rizzo S, Cash P (2005) Anal Biochem 338:263–269
Lee C-J (1983) J Biol Stand. 11:55–64
Lee C-J (1984) J Biol Stand 12:447–450
Lei QP, Shannon AG, Heller RK, Lamb DH (2000) Dev Biol 103:259–264
Lemercinier X, Jones C (1996) Carbohydr Res 296:83–96
Lemercinier XA, Jones C (2006) Carbohydr Res 341:68–74
Leontein K, Lindberg B, Lonngren J (1981) Can J Chem 59:2081–2085
Lindberg B (1990) Thesis, Stockholm University
Lindberg B, Lindquist B, Lonngren J, Powell DA (1977) Carbohydr Res 58:177–186
MacNair JE, Desai T, Teyral J, Abeygunawardana C, Hennessey JP Jr (2005) Biologicals 33:49–58
Merkle RK, Poppe I (1994) Methods Enzymol 230:1–15
Micoli F, Rondini S, Pisoni I, Proietti D, Berti F, Costantino P, Rappuoli R, Szu S, Saul A, Martin LB (2011) Vaccine 29:712–720
Moreau M, Richards JC, Kniskern PJ, Pery MB (1988) Carbohydr Res 182:179–199

Oliver MB, Jones C, Larson TR, Calix JJ, Zartler ER, Yother J, Nahm MH (2013) J Biol Chem 288:21945–21954
Park IH, Pritchard DG, Cartee R, Brandao A, Brandileone MCC, Nahm MH (2007) J Clin Micro 45:1225–1233
Reeves RE, Goebel WF (1941) J Biol Chem 139:511–519
Ricci S, Bardotti A, D'Ascenzi S, Ravenscroft N (2001) Vaccine 19:1989–1997
Richards JC, Kniskern PJ, Perry MB (1984) Can J Biochem Cell Biol 62:666–677
Richards JC, Perry MB (1988) Biochem Cell Biol 66:758–771
Richards JC, Perry MB, Kniskern PJ (1989) Can J Chem 67:1038–1050
Rondel C, Marcato-Romain C-E, Girbal-Neuhauser E (2013) Water Res 47:2901–2908
Rutherford TJ, Jones C, Davies DB, Elliott AC (1991) Carbohydr Res 218:175–184
Rutherford TJ, Jones C, Davies DB, Elliott AC (1994) Carbohydr Res 265:79–96
Slonecker JH, Orentas DG (1962) Nature (London) 194:478–479
Stroop CJM, Xu Q, Retzlaff M, Abeygunawardana C, Bush CA (2002) Carbhohydr Res 337:335–344
Svennerholm L (1957) Biochem Biophys Acta 24:604–611
Swartz LA, Progar JJ, May JC (2000) Biologicals 28:227–231
Sweeney JA, Summer JS, Hennessey JP Jr (2000) Dev Biol (Basel) 103:11–26
Talaga P, Vialle S, Moreau M (2002) Vaccine 20:2474–2484
Thiébaud J, Fanget I, Jaudinaud I, Fourrichon L, Sabouraud A, Talaga P, Uhlrich S (2014) Anal Biochem 453:22–28
Tsai C-M, Gu X-X, Byrd A (1994) Vaccine 12:700–706
Turula VE Jr, Gore T, Singh S, Arumugham RG (2010) Anal Chem 82(5):1786–1792
Vipond C, Mulloy B, Rigsby P, Burkin K, Bolgiano B (2012) MenC is working group. Biologicals 40:353–363
Von-Hunolstein C, Parsi L, Bottaro D (2003) J Biochem Biophys Methods 56:291–296
Walford SN (2010) Proc Int Soc Sug Cane Techno 27:1–15
Warren I, Odin L (1959) J Biol Chem 234:1971
WHO (1966) Technical report, no. 323
WHO (2012) Information and surveillance bulletin, vol 5, Feb 2012
Xie O, Bolgiano B, Gao F, Lockyer K, Swann C, Jones C, Delrieu I, Njanpop-Lafourcade B-M, Tamekloe TA, Pollare AJ, Norheim G (2012) Vaccine 30:5812–5823
Xu Q, Abeygunawardana C, Ng AS, Sturgess AW, Harmon BJ, Hennessey JP (2005) Anal Biochem 336:262–272 (Jan 15)
Zartler ER, Porambo RJ, Anderson CL, Chen LH, Yu J, Nahm MH (2009) J Biol Chem 284:7318–7329

Chapter 8
Glycoconjugate Vaccines

Neil Ravenscroft, Paolo Costantino, Philippe Talaga, Roberto Rodriguez and William Egan

8.1 Introduction to Glycoconjugate Vaccines

8.1.1 From Polysaccharide to Glycoconjugate Vaccines

The first glycoconjugate vaccine to be developed and licensed was for the prevention of invasive *Haemophilus influenzae* type b (Hib) disease in infants and toddlers. The history of the development of this vaccine, in brief follows. Several key observations regarding *H. influenzae* disease were made by Margaret Pittman: namely, (1) that all invasive *H. influenzae* disease was caused by encapsulated organisms; (2) that there were six encapsulated *H. influenza*e serotypes (designated by the letters a through f); (3) that essentially all *H. influenzae* disease was due to type b; and finally, (4) that the capsules were polysaccharides by composition (Pittman 1931; Robbins et al. 1984). The age incidence of invasive Hib disease was subsequently investigated by Fothergill and Wright (1933) who showed that there

N. Ravenscroft (✉)
University of Cape Town, Cape Town, South Africa
e-mail: Neil.Ravenscroft@uct.ac.za

P. Costantino
Novartis Vaccines, Siena, Italy
e-mail: Paolo.Costantino@novartis.com

P. Talaga
Sanofi Pasteur, Marcy l'Étoile, France
e-mail: Philippe.Talaga@sanofipasteur.com

R. Rodriguez
Pfizer, St. Louis, MO, USA
e-mail: Roberto.Rodriguez2@pfizer.com

W. Egan
Novartis Vaccines, Cambridge, MA, USA
e-mail: William.Egan@novartis.com

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6_8

was little disease during the first 3 months of life, with the incidence of disease then rising steeply after to peak at ca. 12 months of age, and to then drop off with little disease occurring after the age of 3–5 years. Additionally, and most importantly, Fothergill and Wright noted that the age incidence of disease was inversely correlated with the "bactericidal power of the blood." Subsequent studies by Alexander, Heidelberger, and Leidy then showed that the increase in the bactericidal power of the blood with increasing age was due to the acquisition of anti-capsular polysaccharide antibodies (Alexander and Leidy 1947; Alexander et al. 1944).

Taken together, the above findings pointed toward the development of a purified Hib capsular polysaccharide vaccine to prevent Hib disease, by analogy to the purified pneumococcal capsular polysaccharide vaccine that was developed by MacLeod and associates and shown to be successful in preventing type specific pneumococcal disease in army recruits (Macleod et al. 1945). A purified Hib capsular polysaccharide vaccine was developed and studied in a large clinical trial of ca. 100,000 infants and young children aged 3 months to 6 years of age in Finland; approximately 50,000 children received the Hib polysaccharide vaccine and 50,000 received a placebo (Peltola et al. 1977). In children >18 months of age, there was no Hib disease among the Hib vaccine recipients and 11 cases among the placebo recipients. However, no protection was seen in children less than 18 months of age. Furthermore, it was observed that the vaccine was not immunogenic in the younger children and infants (but was in the older children). Thus, whereas immunity to Hib disease had been correlated to antibodies against the polysaccharide capsule, children less than 18 months of age, the group for whom the incidence of disease is greatest, did not respond to the purified polysaccharide vaccine.

The solution to this problem stemmed from studies done by Goebel and Avery during the late 1920s and early 1930s. They showed that, for example, an antigen prepared by combining the Type 3 pneumococcal capsular polysaccharide with a protein is capable of inducing in rabbits an active immunity against infection with a virulent culture of Type 3 pneumococcus (Goebel and Avery 1931). As noted by Avery and Goebel in this paper, "Since this carbohydrate alone is non-immunogenic in rabbits, the antibacterial immunity … can be ascribed only to the antigenicity acquired by the polysaccharide through combination with the foreign protein." In similar fashion, 50 years later, Schneerson et al. (1980) developed the basis for a vaccine against Hib disease by conjugating the Hib polysaccharide to diphtheria toxoid. In modern immunological parlance, they had shown that a T-cell independent antigen (the Hib polysaccharide) could be transformed into a T-cell dependent antigen (the polysaccharide–protein conjugate) and serve as the basis for immunizing infants against Hib disease.

The efficacy of a Hib conjugate vaccine was evaluated in a clinical trial of 61,080 children in the Northern California Kaiser Permanente Care Program between February, 1988 and June, 1990 (Black et al. 1991). Vaccine efficacy following either two or three doses of vaccine was 100 %. The efficacy of a second Hib conjugated vaccine was evaluated (Santosham et al. 1991) in a study of 9038 Navajo or Hopi infants between July, 1988 and August, 1990 (the attack rate in certain Native American population is 5–10 times that of the general population).

Vaccine efficacy following two doses of this vaccine was 95 %. Since 1990, the routine use of the Hib conjugate vaccine in the US has led to an approximately 99 % reduction in invasive *H. influenzae* disease in infants and young children.

Subsequently glycoconjugate vaccines against other childhood diseases for which infants do not respond to a plain purified polysaccharide vaccine have been developed and licensed. These include multivalent vaccines for pneumococcal disease (e.g., Prevnar-7 and Prevnar-13, Synflorix) and meningococcal disease (e.g., Menactra, Menveo, Nimenrix). The glycoconjugate vaccine concept is also being extended to a number of additional pediatric vaccines, including a Group B streptococcal vaccine. Furthermore, clinical experience has shown that glycoconjugate vaccines are also superior to the plain polysaccharide vaccines for use in older children, adults, and the elderly.

8.1.2 Licensed Glycoconjugate Vaccines

The first glycoconjugate vaccines licensed were developed in the 1980s against Hib based on the conjugation strategies shown in Fig. 8.1. The saccharide used may be either a long-chain polysaccharide or an oligosaccharide comprising the repeating unit of the Hib capsular polysaccharide,, which is activated and attached to the protein

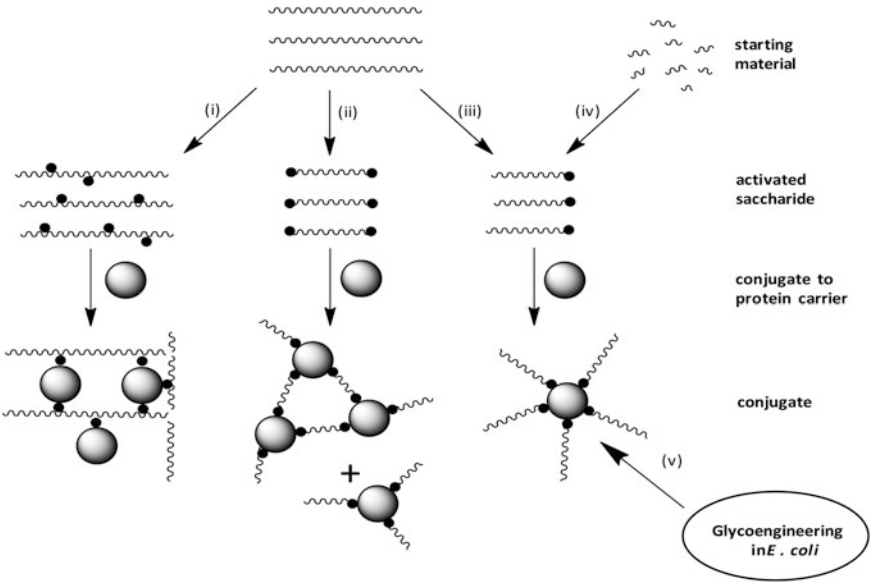


Fig. 8.1 Strategies for the preparation of glycoconjugate vaccines. The purified saccharide may be (*i*) randomly activated; (*ii*) partially fragmented to form active functional groups at both terminals; or (*iii*) activated at only one terminal. A recent approach (*iv*) involves the synthesis of a short saccharide chain which is conjugated directly to the protein. Lastly, it may be prepared by (*v*) biosynthesis. The final structure of the vaccine depends upon the approach adopted. Adapted from Ravenscroft and Feavers (2006), with permission

carrier directly or via a spacer. The polysaccharide used as the starting material in the polysaccharide-based conjugate vaccines is either native or partly size-reduced prior to random activation (i) and attachment, whereas the saccharide component of oligosaccharide-based vaccines can be generated by fragmentation of the polysaccharide to form active functional groups at both terminals (ii), or at only one terminal (iii), which may be used directly or modified prior to conjugation (Lindberg 1999). The third and most recent approach involves synthesis (iv) from readily available chemical precursors to form a small saccharide chain with a terminal, which is conjugated directly to the protein (Verez-Bencomo et al. 2004). Although no licensed products are available, a promising method of glycoconjugate production involves biosynthesis (v), in which *Escherichia coli* (*E. coli*) is engineered to express the carrier protein and the saccharide-repeating unit, which is polymerized then transferred to N-glycosylation sites on the protein (Ihssen et al. 2010; Wacker et al. 2002).

The carrier proteins used for Hib conjugates are based on existing protein vaccines, viz. diphtheria toxoid (DT), tetanus toxoid (TT), CRM197 (a genetically toxoided, single amino acid variant of diphtheria toxin) and an outer membrane complex from *Neisseria meningitidis* group B strain (OMPC). The structure (and immunogenicity) of the derived conjugate depends on the size (and loading) of the antigen, the protein carrier, and the conjugation chemistry employed. Polysaccharide-based vaccines use multiple attachment sites resulting in a cross-linked lattice matrix, whereas oligosaccharide-based vaccines can either have a single terminal attachment site to give a simple monomeric type of structure or be attached through both terminals to form a mixture of monomeric and limited cross-linking structures shown in Fig. 8.1. Although better defined than "traditional" vaccines, these conjugates raise new challenges as biological assays are poorly predictive of immunogenicity in humans, and thus quality control is achieved largely by means of physicochemical methods of analysis instead of bioassays (Brown et al. 2000; Egan et al. 1995; Holliday and Jones 1999). This has the added advantage of reducing the amount of animal testing required (Metz et al. 2002). The advances in bioanalytical technology applied to the molecular characterization of the structurally-defined Hib conjugates and intermediates has greatly facilitated the rapid development, characterization, and licensure of the meningococcal and pneumococcal conjugate vaccines listed in Table 8.1. These vaccines are based on the repeating unit of capsular polysaccharides (Table 8.2) with the specific conjugation approaches adopted depending on the chemical structure (and size) of the antigen, the protein carrier used, and the manufacturer's proprietary technology and experience. The first meningococcal conjugate vaccine was licensed in 1999 against group C disease (in the UK) and a 7-valent pneumococcal conjugate vaccine against serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F in 2000 (in the USA). Since then other meningococcal conjugates containing groups A, Y, W saccharides have been licensed as well as 10- and 13-valent pneumococcal vaccines, with higher valent vaccines in development. Licensure of the 10-valent pneumococcal conjugate vaccine also introduced a new carrier protein, delipidated protein D from *Haemophilus* recombinantly expressed in *E. coli.* and several other carrier proteins are in development (Forsgren and Riesbeck 2008; Prymula et al. 2006). Although, in principle, the

Table 8.1 Glycoconjugate vaccines currently licensed in the US, EU or WHO[a]

Organism	Manufacturer (year licensed)	Saccharide[b]	Protein carrier	Conjugation chemistry
H. influenzae type b	Wyeth (1988)	Hib oligo	CRM 197	Reductive amination
	Merck & Co. (1989)	Hib PS size reduced	OMPC	Thioether linkage
	Sanofi Pasteur (1993)	Hib PS	Tetanus toxoid	Cyanylation chemistry
	GSK (1996)	Hib PS	Tetanus toxoid	Cyanylation chemistry
	Chiron (1996)	Hib oligo	CRM 197	Active ester chemistry
	CIGB (2010)	Hib oligo (synthetic)	Tetanus toxoid	Active ester chemistry
	SIIL (2010)	Hib PS	Tetanus toxoid	Cyanylation chemistry
Meningococcal	Wyeth (1999)	C PS size reduced	CRM 197	Reductive amination
	Novartis (2000)	C oligo	CRM 197	Active ester chemistry
	Baxter (2000)	C De-OAc PS	Tetanus toxoid	Reductive amination
	Finlay Institute	C PS	Mn B OMPs[c]	N/A[d]
	Sanofi Pasteur (2005)	A, C, Y, W PS size reduced	Diphtheria toxoid	Reductive amination/ hydrazide
	Novartis (2008)	A, C, Y, W oligo	CRM 197	Active ester chemistry
	SIIL (2010)	A size reduced PS	Tetanus toxoid	Hydrazide/ reductive amination
	GSK (2012)	A, C, Y, W PS size reduced	Tetanus toxoid	Cyanylation chemistry
Pneumococcal	Wyeth (2000)	14, 6B, 9V, 14, 18C, 19F, 23F PS except 18C (oligo)	CRM 197	Reductive amination
	GSK (2009)	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F size-reduced PS	Protein D; and TT (18C) and DT (19F)	Cyanylation chemistry
	Pfizer (2010)	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F PS except 18C (oligo).	CRM 197	Reductive amination

[a] List excludes combination vaccines against more than one organism
[b] *PS* capsular polysaccharide, *oligo* oligosaccharide
[c] *OMPs* outer membrane proteins
[d] *N/A* not applicable

Table 8.2 Structures of the repeating units of some of the polysaccharides used in vaccine production

Polysaccharide	Repeat unit
Haemophilus influenzae Type b ("PRP")	→3)-β-D-Rib*f*-(1 → 1)-D-Ribitol-(5 → *P*→
Neisseria meningitidis Group A	→6)-α-D-Man*p*NAc(3/4OAc)-(1 → *P*→
N. meningitidis Group C	→9)-α-D-Neu5Ac(7/8OAc)-(2→
N. meningitidis Group W	→6)-α-D-Gal*p*-(1 → 4)-α-D-Neu5Ac(7/9OAc)-(2→
N. meningitidis Group Y	→6)-α-D-Glc*p*-(1 → 4)-α-D-Neu5Ac(7/9OAc)-(2→
Streptococcus pneumoniae Type 1	→3)-D-AAT-α-Gal*p*-(1 → 4)-α-D-Gal*p*A(2/3OAc)-(1 → 3)-α-D-Gal*p*A-(1→
S. pneumoniae Type 3	→3)-β-D-Glc*p*A-(1 → 4)-β-D-Glc*p*-(1→
S. pneumoniae Type 4	→3)-β-D-Man*p*NAc-(1 → 3)-α-L-Fuc*p*NAc-(1 → 3)-α-D-Gal*p*NAc-(1 → 4)-α-D-Gal*p*2,3(S)Py-(1→
S. pneumoniae Type 5	→4)-β-D-Glc*p*-(1 → 4)-[α-L-Pne*p*NAc-(1 → 2)-β-D-Glc*p*A-(1 → 3)]-α-L-Fuc*p*NAc-(1 → 3)-β-D-Sug*p*-(1→
S. pneumoniae Type 6A	→2)-α-D-Gal*p*-(1 → 3)-α-D-Glc*p*-(1 → 3)-α-L-Rha*p*-(1 → 3)-D-Rib-ol-(5 → *P*→
S. pneumoniae Type 6B	→2)-α-D-Gal*p*-(1 → 3)-α-D-Glc*p*-(1 → 3)-α-L-Rha*p*-(1 → 4)-D-Rib-ol-(5 → ***P***→
S. pneumoniae Type 7F	→4)-β-D-Glc*p*-(1 → 3)-[α-D-Glc*p*NAc-(1 → 2)-α-L-Rha*p*-(1 → 4)]-β-D-Gal*p*NAc-(1 →
	→6)-[β-D-Gal*p*-(1 → 2)]-α-D-Gal*p*-(1 → 3)-β-L-Rha*p*(2OAc)-(1→
S. pneumoniae Type 9 V	→4)-α-D-Glc*p*(2/3OAc)-(1 → 4)-α-D-Glc*p*A-(1 → 3)-a-D-Gal*p*-(1 → 3)-β-D-Man*p*NAc(4/6OAc)-(1 → 4)- → β-D-Glc*p*-(1→
S. pneumoniae Type 14	→4)-β-D-Glc*p*-(1 → 6)-[β-D-Gal*p*-(1 → 4)]-β-D-Glc*p*NAc-(1 → 3)-β-D-Gal*p*-(1→
S. pneumoniae Type 18C	→4-)β-D-Glc*p*-(1 → 4)-[α-D-Glc*p*(6OAc) (1 → 2)][Gro-(1 → ***P***→3)]-β-D-Gal*p*-(1 → 4)-α-D-Glc*p*-(1 → 3)-β-L-Rha*p*-(1→
S. pneumoniae Type 19A	→4)-β-D-Man*p*NAc-(1 → 4)-α-D-Glc*p*-(1 → 3)-α-L-Rha*p*-(1 → ***P***→
S. pneumoniae Type 19F	→4)-β-D-Man*p*NAc-(1 → 4)-α-D-Glc*p*-(1 → 2)-α-L-Rha*p*-(1 → ***P***→
S. pneumoniae Type 23F	→4)-β-D-Glc*p*-(1 → 4)-[α-L-Rha*p*-(1 → 2)]-[Gro-(2 → ***P***→3)]-β-D-Gal*p*-(1 → 4)-β-L-Rha*p*-(1→

AAT is 2-acetamido-4-amino-2,4,6-trideoxygalactose, *Gro* is glycerol, *Pne* is 2-acetamido-2,6-dideoxytalose, Sug*p* is 2-acetamido-2,6-deoxyhexose-4-ulose, and *P* is phosphate in a phosphodiester linkage

carrier protein can act as a protective antigen in its own right, no glycoconjugate vaccines have yet been licensed for such dual use. As described in Sect. 8.7.1, there are many other bacterial vaccines in development; these are based on surface

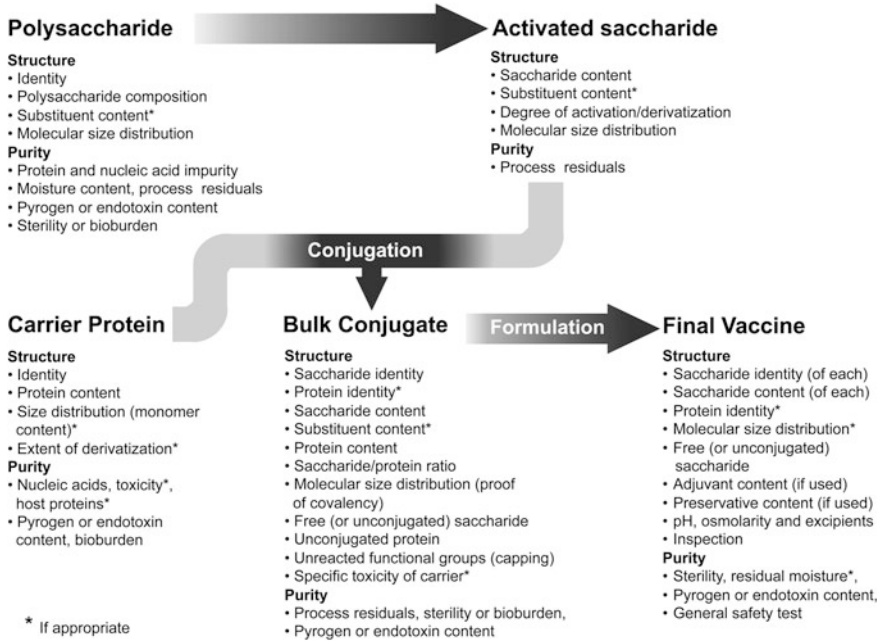


Fig. 8.2 Control tests applied to conjugate vaccines. Control tests typically applied at each stage during the manufacture of the conjugate vaccines are shown. Details are published in World Health Organization recommendations, European Pharmacopoeia monographs and a United States Pharmacopeia chapter. Adapted from Ravenscroft and Feavers (2006), with permission

carbohydrates of pathogens and utilize the glycoconjugate technology platforms established for Hib, meningococcal, and pneumococcal vaccines.

Quality control and licensure of glycoconjugate vaccines is based on the bio-analytical outline first described in the WHO Recommendations for *H. influenzae* type b conjugate vaccines (Anonymous 2000) and the European Pharmacopoeia (Ph. Eur.) monograph 1219. WHO Recommendations for meningococcal group C and group A conjugate vaccines were published in 2004 and 2006, respectively (Anonymous 2004, 2006), with a Ph. Eur. monograph 2112 for group C vaccines. The WHO Recommendations for pneumococcal conjugate vaccines were published in 2005 (Anonymous 2005) and the serological assessment updated in 2009 (Anonymous 2009), while the Ph. Eur. monographs 0966 and 2150 describe the criteria for the pneumococcal polysaccharide and conjugate vaccines, respectively. A new United States Pharmacopeia (USP) chapter <1234> under Vaccines for Human Use (Anonymous 2013a) describes the best practices for production, conjugation, and characterization of polysaccharide and glycoconjugate vaccines; accompanying guidelines on appropriate analytical methods are in development. The analysis of glycoconjugate vaccines described by these Recommendations and Pharmacopoeia can be divided into various subsections as summarized in Fig. 8.2.

This chapter will focus on the strategies and analytical methodologies currently applied and in development for the licensure of glycoconjugate vaccines.

8.2 Polysaccharide

Currently licensed conjugate vaccines are all derived from the corresponding polysaccharides discussed in this chapter and are required to meet the quality criteria already established for polysaccharide vaccines.

8.2.1 Polysaccharide Production

Capsular polysaccharides are prepared by fermentation of bacterial cultures followed by isolation and purification using a combination of selective precipitation, diafiltration, and/or chromatographic steps depending on the chemical nature of the polysaccharide. The final purification step consists of buffer exchange and filtration or by ethanol precipitation, recovery, and drying, followed by storage of purified polysaccharide as a dry powder or as a frozen solution. The stability of the polysaccharide under specified storage conditions needs to be demonstrated using some of the techniques described in this section. The purified polysaccharides are subjected to biochemical testing to show identity, purity, and molecular size of the antigens according to criteria established by WHO and Pharmacopoeia guidelines. In addition to the polysaccharide specific release assays, the polysaccharide must contain only low amounts of impurities, such as protein, nucleic acid, and endotoxin (Fig. 8.2).

8.2.2 Polysaccharide Structure and Identity

Antigen structure/identity has traditionally been determined using a combination of wet chemical methods for characteristic carbohydrate (sialic acid, uronic acid, hexosamine, methylpentose) and other (nitrogen, phosphorus, *O*-acetyl) components (Table 8.3) and an identity test. Immunological identity methods may involve immunoprecipitation, immunoelectrophoresis, or enzyme linked immunosorbent (ELISA) assays, but require access to highly specific antisera that are able to distinguish between closely related polysaccharide antigens. The selective nature of the tests means that although they may confirm the presence of the antigen tested for, they give no information regarding other material that may be present. Given that the molecular structure of the polysaccharides used for vaccine production have been elucidated, structural techniques such as nuclear magnetic resonance (NMR) spectroscopy can demonstrate identity and give an indication of purity. Proton NMR spectra contain characteristic signals depending on the electronic environment of the

Table 8.3 Colorimetric and composition assays applied to polysaccharides, intermediates and conjugate vaccines

PS antigen	Colorimetric assays[a]	Composition assays				
		Acid hydrolysis/ HPAEC	Acid hydrolysis/fluorophore labeling-HPLC	HF hydrolysis/ HPAEC	Base hydrolysis/ HPAEC	Methanolysis/ GC-FID/MS
Hib	Ribose[b]	Ribitol		Phosphate	PRP monomer	Ribitol
	Phosphate[c]					
Mn A	Phosphate	ManN-6-P		Phosphate	*O*-acetyl	
Mn C	NeuNAc[d]	NeuNAc	NeuNAc		*O*-acetyl	
	O-acetyl[e]					
Mn Y	NeuNAc	Glc, NeuNAc	Neu5Ac		*O*-acetyl	
	O-acetyl					
Mn W	NeuNAc	Gal, NeuNAc	Neu5Ac		*O*-acetyl	
	O-acetyl					
Pn (serotype specific)	Phosphate	Alditols	Methylpentoses Hexoses	Phosphate	*O*-acetyl	Alditols
	Uronic acid[f]	Methylpentoses Hexoses	Hexosamines			Methylpentoses Hexoses
	Hexosamines[g]	Hexosamines Uronic acids				Hexosamines Uronic acids
	Methylpentose[h]	Pyruvate (Pn 4)				
	O-acetyl					

[a] Assay details and references are contained in the corresponding WHO TRS and Pharmacopoeia
[b] Bial assay (Ashwell 1957)
[c] Molybdate assay (Chen et al. 1956)
[d] Hestrin (1949)
[e] Resorcinol assay (Svennerholm 1957)
[f] Carbazole assay (Dische 1947)
[g] Dimethylaminobenzaldehyde assay (Elson and Morgan 1933)
[h] Cysteine sulfuric acid assay (Dische and Shettles 1948)

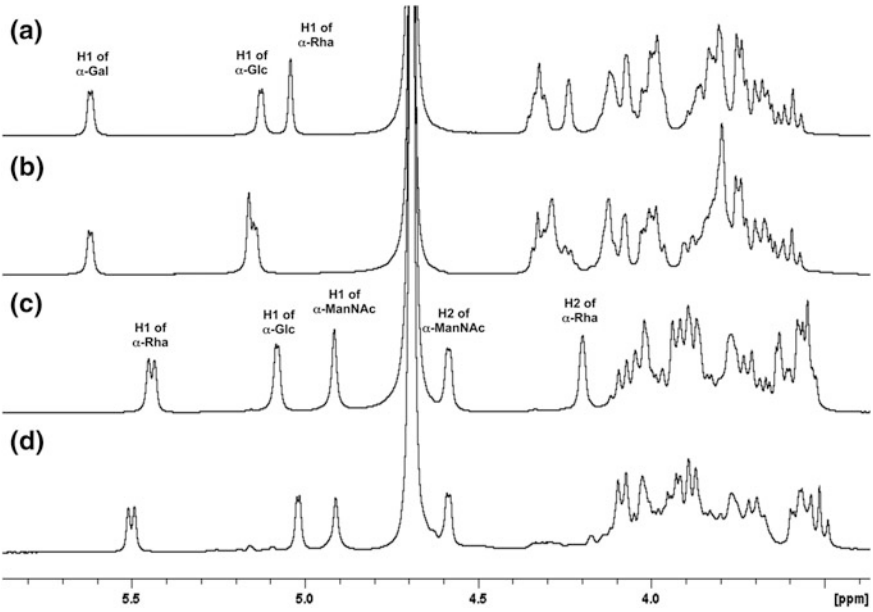


Fig. 8.3 Partial ^{1}H NMR spectra of the anomeric and ring regions of the spectra of closely related pneumococcal serotype polysaccharides that have the same composition but differ in a single linkage position. **a** Pn 6A and **b** Pn 6B; **c** Pn 19A and **d** Pn 19F. See Table 8.2 for the structures of the polysaccharide repeating units

protons present and their relative intensities can confirm the proportions of different sugar residue types and substituents (such as *N*- or *O*-acetyl) present in the polysaccharide (Lemercinier and Jones 1996, 2000; Jones and Lemercinier 2002; Jones and Ravenscroft 2008; Jones 2005; Jones et al. 2000). Quantification can be achieved by comparison of these intensities to that of an added internal standard (Abeygunawardana et al. 2000). The sensitivity and selectivity of 1D proton NMR permits differentiation between two closely related polysaccharides that differ due to a single linkage such as Pn 6A/6B (→3)-D-Rib-ol/→4)-D-Rib-ol) and Pn 19A/19F (→3)-α-L-Rha*p*/→2)-α-L-Rha*p*) as shown in Fig. 8.3, or only in configuration at a single carbon as between Mn W and Y (→6)-α-D-Gal*p*/→6)-α-D-Glc*p*).

The ability to characterize molecules at the atomic level has allowed NMR spectroscopy to play a leading role in the structural identification of several new pneumococcal serotypes discovered within serogroups, which were considered to be well characterized by serological methods (Calix et al. 2011, 2012; Oliver et al. 2013; Park et al. 2007; Zartler et al. 2009). NMR data identifying the position of O-acetylation of polysaccharide vaccines have been published; this allows for determination of the position and extent of O-acetylation of polysaccharide lots, which may be important for antigenicity and for the subsequent activation reaction if based upon hydroxyl groups (see Sect. 8.3.1). For polysaccharides that contain variable O-acetylation resulting in spectral complexity that hampers the use of

NMR as a polysaccharide identity test, a second spectrum can be recorded after simplifying the spectrum by performing base-catalyzed de-O-acetylation in the NMR tube. This approach has been reported for meningococcal polysaccharides, but could also be applied to pneumococcal serotypes such as Pn 1, 7F, 9V, and 18C (Jones and Lemercinier 2002). Finally, NMR analysis can also be used to identify and potentially quantify other organic materials present in the polysaccharide preparation such as contaminants and process residuals as well as product residuals such as cell wall polysaccharide (CWPS) for pneumococcal polysaccharides (Abeygunawardana et al. 2000). CWPS is composed of a tetrasaccharides repeating unit joined by ribitol phosphodiester bonds with phosphocholine substituents. Quantification has been reported by use of ^{1}H or ^{31}P NMR analysis of the phosphocholine moiety or by ribitol analysis (GC-MS and HPAEC-PAD), depending on the serotype (Talaga et al. 2001; Xu et al. 2005a). Residual CWPS may be chemically linked to the capsular polysaccharide (bound) or present by itself (free); these were quantified by two methods: labeling CWPS with a fluorescent tag followed by SEC-HPLC-RI/FL and an NMR method based on self-diffusion rates (Xu et al. 2005a). The associated peptidoglycan may be quantified by the same techniques. Capillary zone electrophoresis (CZE) has been shown to be a robust technique for the separation and identification of a mixture of group A, C, Y, and W polysaccharides (Lamb et al. 2005); additionally quantification could be achieved without the need for colorimetric assays or composition analysis (Sect. 8.2.3).

8.2.3 Polysaccharide Composition Analysis

Hib, meningococcal, and pneumococcal polysaccharide composition (and quantitation) is determined by the use of colorimetric assays as shown in Table 8.3, assay details and expected limits based on dry weight are described in the corresponding WHO and Pharmacopoeia guidelines.

Chromatographic methods offer greater specificity and sensitivity than colorimetric assays, and can be applied to quantify saccharide and nonsugar components of the antigens throughout the conjugation manufacturing process. Depolymerization to monomers is achieved by acid or base treatment with quantitation of specific analytes by use of the chromatographic techniques outlined in Table 8.3. Suitable polysaccharide reference materials or monosaccharides are required and the depolymerization conditions are product-specific and may not be quantitative for all components. Hydrolysis is always a compromise of cleavage of glycosidic bonds and subsequent degradation of the released monomer, thus hydrolysis conditions that are strong enough to release the hexoses from Mn Y and W cause concomitant degradation of the labile sialic acid. This problem is exacerbated for multiresidue pneumococcal polysaccharides that contain both acid-resistant (e.g., uronic acids, amino sugars) and acid-labile keto and deoxy sugar monomers. Phosphodiester linkages can be selectively hydrolyzed by use of hydrofluoric acid (HF). Liquid chromatography (LC) permits direct analysis of the analytes, whereas gas–liquid

chromatography (GC) and some LC methods require prior derivatization. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) methods have been described for the quantitation of Hib [as monomer (Haan et al. 2013; Lei et al. 2000a; Sturgess et al. 1999; Tsai et al. 1994) or ribitol (Bardotti et al. 2000)], meningococcal group A [as mannosamine-6-phosphate (Ricci et al. 2001)], group C (as sialic acid) and groups Y and W [as glucose and galactose, respectively (Bardotti et al. 2005, 2012)], as well as pneumococcal polysaccharides (Yu Ip et al. 1992; Talaga et al. 2002) and the associated CWPS [as ribitol (Talaga et al. 2001)]. A recent method uses selective trifluoroacetic acid hydrolyses and a single HPAEC method to analyze multivalent Mn A, C, Y and W vaccines (Cook et al. 2013). Ionic components of polysaccharide vaccines such as uronic acids, phosphate and acetate can also be analyzed by HPAEC with conductivity detection (HPAEC-CD) as described by Kao and Tsai (2004). Capillary ion electrophoresis with indirect detection was used as an alternative method to ion chromatography for the analysis of *O*-acetate groups in Pn serotypes 9V and 18C (Hepler and Yu Ip 1994). Additional specific methods for sialic acid quantitation have been reported including HPAEC-PAD analysis of the underivatized methyl glycosides (Turula et al. 2004) and HPLC of a fluorophore-labeled derivative (Hara et al. 1987). Two approaches for the composition analysis of pneumococcal vaccines that use derivatization are: reversed phase HPLC of fluorophore-labeled sugars (Canaán-Haden et al. 2006) and gas chromatography with mass spectrometric detection (GC-MS) performed after methanolysis, re-N-acetylation and trimethylsilylation (Kim et al. 2005). Even though each monosaccharide results in more than one peak, the high efficiency of separation afforded by GC together with confirmation of peak assignment and peak purity by analysis of the mass spectra makes this hyphenated technique superior to current liquid chromatographic methods.

8.2.4 Polysaccharide Molecular Size Distribution

The molecular size distribution of the purified polysaccharide reflects manufacturing consistency and indicates the dimensional integrity of the polysaccharide; this is important for those polysaccharides that are susceptible to hydrolysis (for example meningococcal group A and pneumococcal serotypes 6A and 19A). The potency of polysaccharide vaccines requires that they have a molecular size greater than a specific value defined in the WHO and Pharmacopoeia guidelines. The methods are based on gel filtration using Sepharose columns with collection and colorimetric analysis of fractions or refractive index detection and the results are reported as the distribution coefficient (K_D) determined from the main peak of the elution curve or as the percentage of material eluting before a defined K_D cut-off value. However, depending on the conjugation strategy employed, these minimum size requirements for the polysaccharide may not apply and time-consuming soft gel analysis has generally been replaced by high performance size-exclusion chromatography (SEC-HPLC) with refractive index (RI) or multi-angle laser light scattering (MALLS)/RI

detectors. SEC-HPLC/MALLS/RI is used as a release test for pneumococcal polysaccharides; the absolute weight-average molecular mass (Mw) determined for each serotype is required to meet minimum release specifications for that particular serotype (Bednar and Hennessey 1993). This method has been modified for analysis of the polyvalent pneumococcal polysaccharide vaccine (PV23) by adding off-line rate nephelometric detection (SEC-HPLC/RN) to yield the relative molecular size of each serotype; a study aligning the absolute and relative molecular size specifications for a polyvalent polysaccharide vaccine has been published (Sweeney et al. 2000; MacNair et al. 2005). Stability and forced degradation studies showed that a decrease in relative antigenicity was accompanied by a decrease in molecular size for some polysaccharides (Pn 19A, 19F and 5) but not for Pn 1, 9V, and 18C; this was attributed to losses or shifts of *O*-acetyl groups. However, a decrease in relative antigenicity could not be definitively linked to loss of immunogenicity for these serotypes (McNeely et al. 1998; Sweeney et al. 2000). Lastly, polysaccharides containing chromophores can also be analyzed by soft gels (Munoz et al. 2000) or SEC-HPLC with ultraviolet (UV) detection (von Hunolstein et al. 2003).

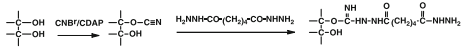
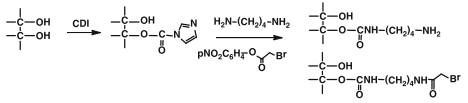
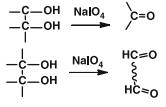
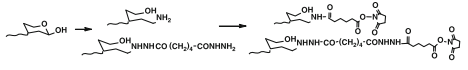
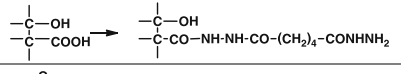
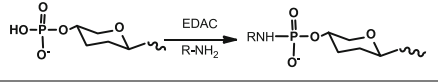
8.3 Saccharide Intermediate

The saccharide intermediate is defined as the purified polysaccharide that has been modified by chemical reaction or physical process and activated in preparation for conjugation to the carrier protein to form the glycoconjugate vaccine. In order to ensure a reproducible conjugate product by means of consistent application of the appropriate conjugation stoichiometry, the saccharide intermediate needs to be fully characterized in terms of structure, active groups, and size distribution. If stored before use, the stability and structural integrity of the saccharide intermediate and active groups needs to be demonstrated.

8.3.1 Strategies for Saccharide Modification/Activation/Processing

Different activation/derivatization strategies can be applied to polysaccharides in order to make them suitable for covalently coupling to the carrier protein; the activation strategy employed depends on the polysaccharide structure and has been the subject of several reviews (Bundle 2011; Costantino et al. 2011; Farkaš and Bystrický 2010; Zou and Jennings 2009). The aim is to achieve a balance between sufficient activation of the saccharide to ensure conjugation to the carrier protein in reasonable yield, while having a low enough degree of activation such that the structural integrity of the carbohydrate antigen is maintained. Table 8.4 provides a summary of the common strategies for saccharides activation/derivatization with the associated quality control testing indicated.

Table 8.4 Common saccharide activation strategies and analytical testing

Functional group	Saccharide activation chemistry	Saccharides	Analytics[a]
Hydroxl activation		General	The active cyanate ester generally not isolated. Colorimetric and HPLC assay for hydrazide introduced
		General	The active imidazolylurethane generally not isolated. Colorimetric amino group assay before and after bromoacylation indicates derivatization. NMR analysis provides degree and position of derivatization and saccharide integrity (Xu et al. 2005b)
Hydroxl diol: cleavage and activation (yields terminal or in-chain aldehydes)		Diols may need to generated after partial removal of substituents	Colorimetric assay for aldehydes introduced (D'Ambra et al. 1997). Location and quantification by NMR analysis (Jones and Ravenscroft 2008) and methylation/linkage analysis (Kim et al. 2006a)
Terminal reducing end (aldose or ketose)		Generated by hydrolysis (e.g. acid, peroxide)	Activated saccharide isolated. Colorimetric assay for active ester
			Activated saccharide-ADH isolated or further derivatized with an active ester. Colorimetric assay for hydrazide/active ester introduced
Carboxyl activation		Uronic or keto-acid containing	Activated saccharide-ADH isolated. Colorimetric assay for hydrazide introduced
Phosphate		Phosphate containing	Activated saccharide can be directly coupled to carrier or derivatized with aminolinker or ADH

[a] Where applicable this requires quantification and evaluation of the integrity of the saccharide (and labile substituents) by use of colorimetric, composition or NMR analysis described in Sect. 8.2

In a general approach applicable to most polysaccharides, the hydroxyl groups are first reacted with cyanogen bromide/CDAP or carbonyldiimidazole to form active esters. These active intermediates can then be reacted in situ with adipic acid dihydrazide (ADH) or other bifunctional amines to introduce an amino linker, in some cases the activated/derivatized saccharide is isolated and represents an intermediate of the glycoconjugate vaccine manufacturing process. Alternatively the saccharide active esters can be reacted directly with the protein carrier and no such intermediate is isolated or analyzed (Shafer et al. 2000). The activation chemistry described may be performed on the native polysaccharide or the size-reduced product. Size-reduction can be achieved by chemical or mechanical treatment (e.g., microfluidization) and yields a saccharide that is less viscous and more amenable to chemical manipulation and processing than the native polysaccharide (Kniskern et al. 2010).

A second general strategy based on saccharide hydroxyl groups involves periodate ($NaIO_4$) oxidation of vicinal hydroxyls to generate aldehydes that are readily conjugated to the amino groups of protein carriers. The effect of the periodate treatment depends on the polysaccharide structure and can be controlled stoichiometrically or kinetically. For Hib and Mn C, which both contain in-chain diols from ribitol and the exocylic chain, respectively, periodate treatment causes simultaneous depolymerization and yields terminal aldehydes (Anderson et al. 1986), whereas for other polysaccharide structures containing several residues per repeating unit, multiple oxidation sites are available and these will be oxidized at different rates depending on the relative orientation of the diols (*cis* vs. *trans*) and whether the residue is an alditol, terminal, or branched sugar. The complexity of periodate oxidation of pneumococcal polysaccharides has been described by Kim et al. 2006a, b, together with a method for determining the location and order of activation by methylation/linkage analysis. For polysaccharides containing diols that are not available due to the presence of *O*-acetyl groups (Pn 1) or pyruvate (Pn 4), these substituents can be partially removed prior to periodate treatment (Prasad 2011). The saccharides containing aldehyde groups can be covalently coupled to lysine residues and the N-terminal amino groups of the carrier protein by reductive amination in the presence of sodium cyanoborohydride ($NaBH_3CN$) or other reducing agents selective for Schiff bases. Alternatively, the aldehydes generated can be reacted with hydrazide-derivatized proteins as reported for the preparation of meningococcal A and C tetanus toxoid conjugates (Lee et al. 2009; Silveira et al. 2007).

Controlled acid hydrolysis of the native polysaccharides produces oligosaccharides that can be specifically derivatized at their reducing end groups by a sequence of steps, which leads to an active ester function. The activated oligosaccharide can be isolated and characterized before conjugation. This approach requires an acid-labile linkage and has been used for Hib and multivalent Mn vaccines (Costantino et al. 1992, 1999; Ravenscroft et al. 2000) and is applicable to some pneumococcal polysaccharides (Porro 1994). Hydrogen peroxide has also been used to generate meningococcal oligosaccharides and the reducing end groups reacted with ADH to form the hydrazide-derivatized saccharide intermediate (Cai

et al. 2004). Some bacterial polysaccharides possess carboxyl or phosphate groups that might be used for introduction of an amino linker using a carbodiimide-mediated chemistry (Beuvery et al. 1983a, b). In all cases, the incorporated amino functional group is suitable for covalent coupling to carboxyl groups of the carrier protein to obtain the desired glycoconjugate (Szu et al. 1987; Fattom et al. 1995). Alternatively, the amino-polysaccharide can be further derivatized to obtain a bromo-acyl or a maleimido function amenable for coupling with a thiol group present or previously incorporated into the carrier protein (Marburg et al. 1986).

8.3.2 *Degree of Activation/Derivatization and Antigen Integrity*

To ensure a consistent conjugation stoichiometry, and therefore a consistent manufacturing process, the degree of activation/derivatization of the saccharide intermediates should be determined. This requires appropriate methods for determination of the amount of the active/functional group introduced (e.g., amino group, aldehyde or active ester) per repeating unit polysaccharide; colorimetric, chromatographic and NMR methods may be used. Colorimetric assays are simple and have been applied to quantitate amino and hydrazide groups (Snyder and Sobocinski 1975; Qi et al. 1988) as well as reducing end groups and aldehydes (D'Ambra et al. 1997; Park and Johnson 1949; Sawicki et al. 1961). Chromatographic methods for the quantification of free and saccharide-linked amino spacer by fluorescent labeling followed by RP-HPLC or capillary zone electrophoresis (CZE) have been reported; these are more sensitive and faster than the traditional gel filtration and colorimetric assays previously used (Adam and Moreau 2000). Calculation of the degree of activation/derivatization also requires measurement of total saccharide; this can be determined using composition or colorimetric assays (Table 8.3) or by use of a general carbohydrate assay such as phenol/sulfuric (Dubois et al. 1956) or anthrone (Turula et al. 2008). Alternatively, the degree of activation/derivatization can be determined from a single assay by use of NMR spectroscopy. This has been described for a derivatized *H. influenzae* type b polysaccharide intermediate generated by hydroxyl activation, reaction with excess butanediamine followed by acetylation with bromoacetyl chloride. Use of an internal standard (dimethyl sulfoxide), permitted quantitative analysis of each of these reagents with respect to the saccharide, and moreover elucidated the sites of derivatization, thus replacing several labor-intensive chromatographic and colorimetric tests (Xu et al. 2005b). Similarly, NMR can fully characterize oligosaccharide-based intermediates; however, its use may be limited when applied for the location and quantitation of low levels of periodate activation at multiple sites on polysaccharides (Kim et al. 2006a). For conjugation strategies involving polysaccharide fragmentation resulting in oligosaccharides with terminal activation (Fig. 8.1, approaches (ii) and (iii)), the molecular size correlates to the degree of activation. Size analysis can be performed by size exclusion or anion

exchange chromatography (see Sect. 8.3.3) and used for monitoring the activation/depolymerization reaction. The average degree of polymerization (DP) follows from the ratio of end groups to sugar chain and can be determined by colorimetric assays, chromatography (HPAEC-PAD after end group reduction and hydrolysis to monomers) or NMR spectroscopy (Bardotti et al. 2005; D'Ambra et al. 1997; Ravenscroft et al. 1999).

When activation is performed randomly along the saccharide chain, the degree of activation must be low to ensure that the structural integrity of the carbohydrate antigen is maintained, but sufficient to permit conjugation to the carrier protein. Therefore, in addition to determining the degree of the activation, it is important to verify that the activation/derivatization treatment has not altered the structure of the saccharide and that the protective epitopes have been retained. The potential structural changes are antigen and conjugation chemistry specific and may include the formation of new epitopes. For example, reductive amination may also reduce the keto sugar present in pneumococcal serotype 5 polysaccharide (to *N*-acetylated quinovosamine and *N*-acetylated fucosamine) (Mistretta et al. 2010). Cyanylation chemistry may hydrolyse phosphodiester linkages or results in some loss or migration of *O*-acetyl groups due the basic conditions employed, alternatively it may also modify antigen amino groups present (2-acetamido-4-amino-2,4,6-trideoxygalactose in serotype Pn 1). Carbodiimide chemistry generates a stable by-product *N*-acylurea, which has been investigated by Beuvery et al. (1985); it has been reported to have immunomodulatory properties (Matsumoto et al. 2004; Ruiz-Perez et al. 2003). These and other potential modifications of saccharide structure can be assessed using physicochemical and immunochemical methods. Colorimetric and composition assays (Table 8.3) of the saccharide intermediate compared to the polysaccharide can confirm maintenance of the correct chemical composition i.e., that there has been no loss of sugar residues and other components such as phosphate, acetate, and pyruvate sidegroups that may be immunodominant. To demonstrate the information provided by these structural tools, HPAEC-PAD and GC-MS composition profiles have been presented for native, fragmented, aminated, and conjugated Pn14 and 18C (Talaga et al. 2002), and native, periodate activated and conjugated Pn 7F, respectively (Kim et al. 2005). As shown in Fig. 8.4, the similarity of the chromatograms obtained for the parent polysaccharide, and the corresponding intermediates confirmed that there were no detectable changes in the antigen chemical composition during the conjugation process.

Similarly, NMR analysis has been used to track antigen integrity from the starting polysaccharide, through several key intermediates (Fig. 8.5) to the final conjugate (Bardotti et al. 2008; Ravenscroft 2000; Ravenscroft et al. 2000) and reveals subtle changes in saccharide structure including the location and extent of O-acetylation, which may be altered during the activation steps (Jones and Ravenscroft 2008). CZE used for identification and quantitation of meningococcal polysaccharides (Lamb et al. 2005), was also shown to be applicable to the size-reduced saccharides and to the ADH-derivatized intermediates.

Immunochemical methods can also be used to verify antigenic integrity of the saccharide after chemical modification, techniques such as immunoelectrophoresis,

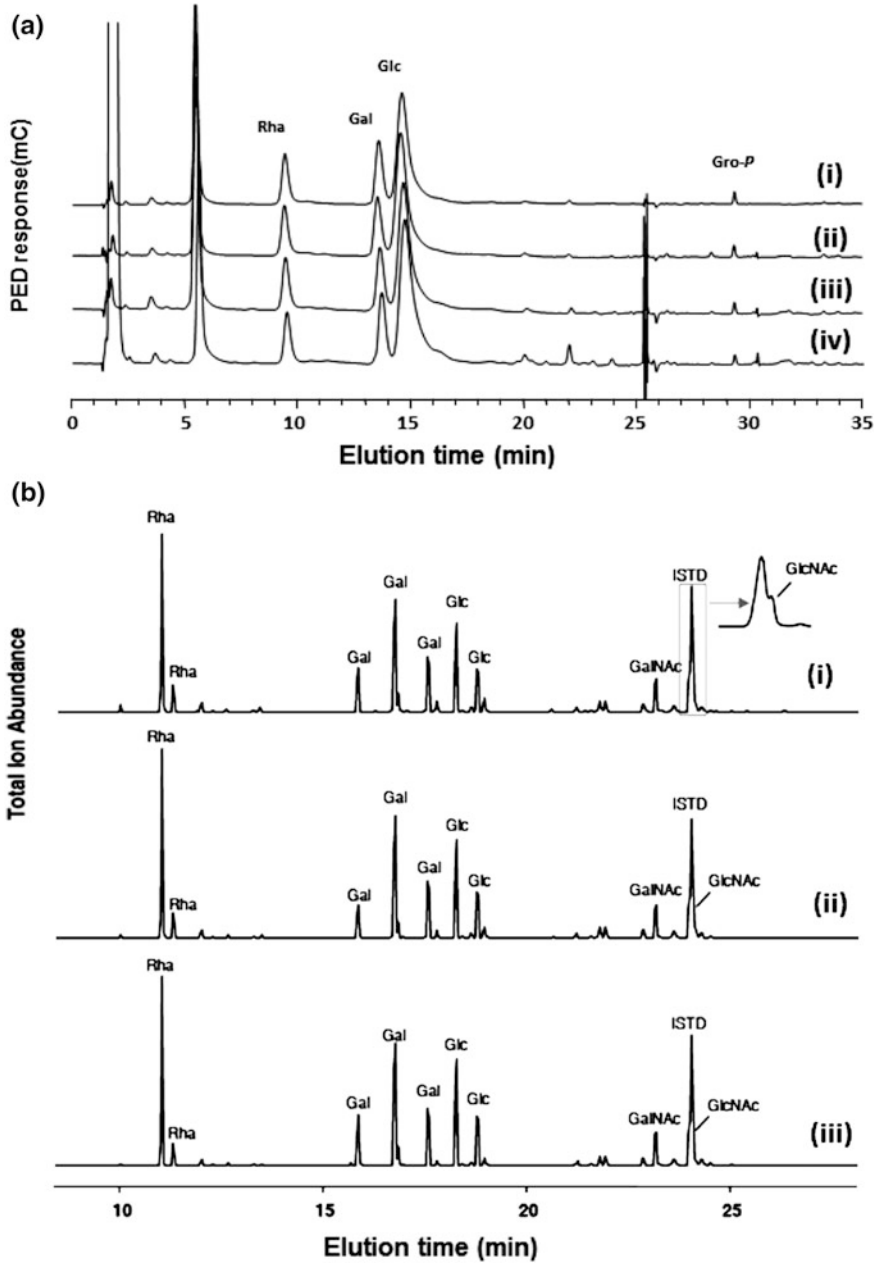


Fig. 8.4 Tracking saccharide composition from polysaccharide through to conjugate using HPAEC-PAD (**a**) and GC-MSD (**b**). **a** HPAEC-PAD chromatograms for Pn 18C following acid hydrolysis of the purified polysaccharide (*i*), size-reduced saccharide (*ii*), activated saccharide (*iii*) and conjugate (*iv*). Reproduced with permission (Talaga et al. 2002). **b** GC-MSD total ion chromatograms for Pn 7F following methanolysis of the purified polysaccharide (*i*), activated saccharide (*ii*) and conjugate (*iii*). Reproduced with permission (Kim et al. 2005). See Table 8.2 for the structures of the polysaccharide repeating units

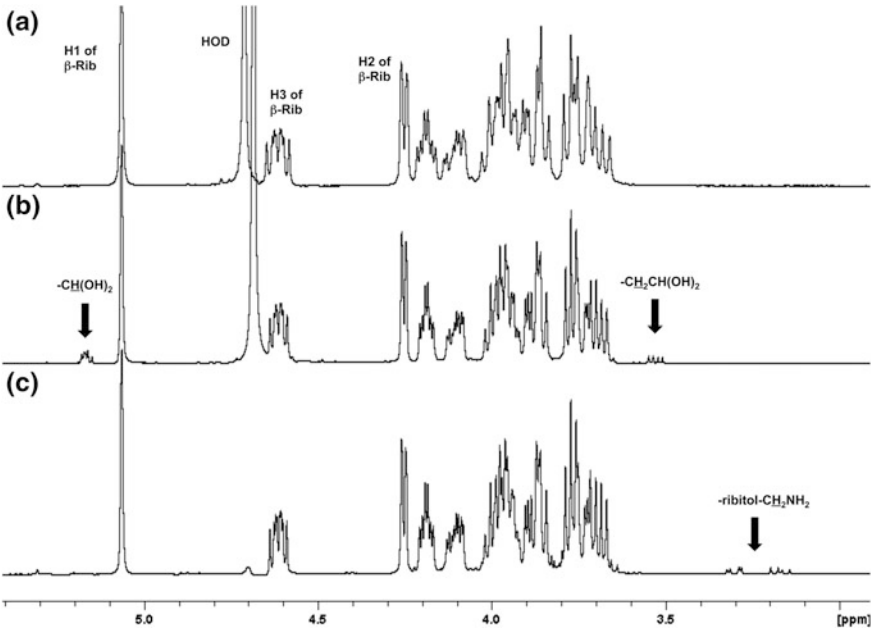


Fig. 8.5 ^{1}H NMR spectra of Hib polysaccharide (**a**) and Hib saccharide intermediates. **b** Activated Hib saccharide generated by periodate oxidation (Fig. 8.1, approach (ii)), the resonances due to the hydrated aldehyde and an adjacent methylene proton are labeled. **c** Hib saccharide intermediate generated by acid hydrolysis, size selection and reductive amination (Fig. 8.1, approach (iii)), the methylene resonances due to the aminated ribitol are labeled

rate nephelometry (RN), competitive ELISA, and surface plasmon resonance, have been applied. One approach uses an index of antigenicity, which involves a comparison of the concentration calculated immunologically by rate nephelometry with the concentration obtained chemically or physically; the index was used to evaluate pneumococcal polysaccharide size-reduced intermediates (Marburg et al. 1997, Sweeney et al. 2000).

8.3.3 Saccharide Molecular Size Distribution

The molecular size (and DP) of the saccharide intermediates depend on the particular manufacturing process used and can affect the consistency of the conjugation process. Although the saccharide molecular size does not strictly relate to the immunogenicity of the final product, it is important to control its distribution because it reflects manufacturing consistency, indicates the structural integrity of the saccharide intermediate and will define the conjugate formed. Suitable methodologies for molecular size profiling of polysaccharides are size exclusion chromatography (SEC) coupled to UV, refractive index, viscometry, and MALLS detectors (Fig. 8.6a,

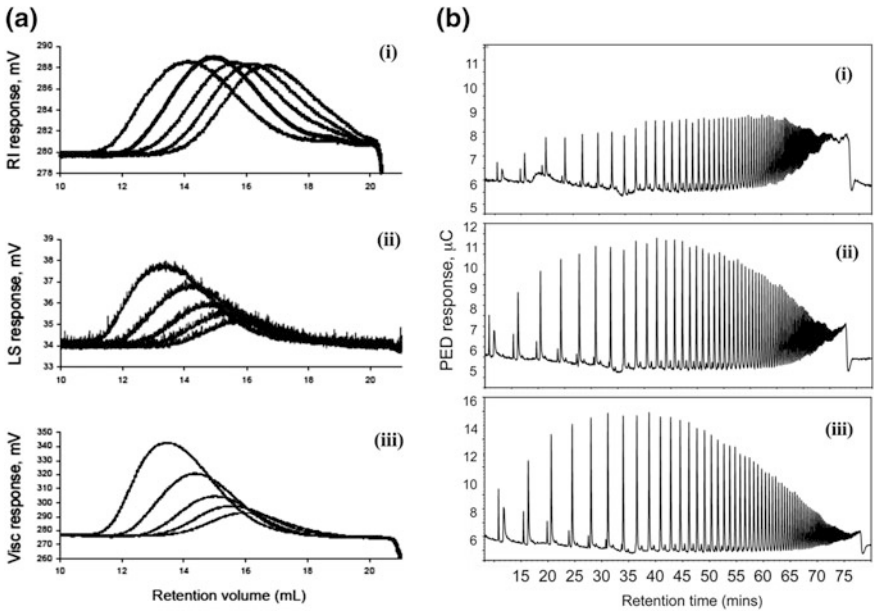


Fig. 8.6 Tracking polysaccharide depolymerization by use of SEC-HPLC (**a**) and HPAEC-PAD (**b**). **a** SEC-HPLC chromatograms with detection using refractive index (*i*), light scattering (*ii*) and viscometry (*iii*). **b** HPAEC-PAD profiles following the mild acid hydrolysis of Mn W polysaccharide at 2.5 (*i*), 4.5 (*ii*) and 6.5 h (*iii*). Reproduced with permission from Tomei et al. (2009)

Sect. 8.2.3), whereas oligosaccharides can be analyzed by DP analysis (based on total and end groups determination), HPAEC-PAD, or ^{1}H NMR spectroscopy. ^{31}P NMR spectroscopy can also be used for phosphodiester-containing saccharides, such as Hib, Mn A, and some pneumococcal serotypes (see Table 8.2). HPAEC–PAD profiles can be used to monitor the mild acid hydrolysis of meningococcal polysaccharides to oligosaccharides and track the sizing and activation intermediates (Fig. 8.6b); this demonstrates the precision with which the oligosaccharide-based conjugates can be characterized (Proietti et al. 2005; Tomei et al. 2009).

8.4 The Carrier Protein

The carrier protein is defined as the protein to which the saccharide is covalently linked for the purpose of eliciting a T-cell-dependent immune response to the polysaccharide antigen. This response is developed early in life and leads to immunological memory and boosting of the response by further doses of the vaccine; thus, conjugate vaccines are suitable for infant immunization. The most widely used carrier proteins are related to bacterial toxins that are detoxified by chemical or genetic means. Saccharides can be covalently attached to proteins,

although activation is required. Sometimes the carrier protein is also activated or derivatized to create compatible reactive groups. As for the saccharide intermediate, the quality of the protein carrier needs to be demonstrated in order to ensure the reproducible production of the glycoconjugate vaccine.

8.4.1 Licensed Protein Carriers

Five carrier proteins are currently used for licensed conjugate vaccines: diphtheria toxoid (DT), tetanus toxoid (TT), CRM197, *Haemophilus* protein D (PD), and the outer membrane protein complex of serogroup B meningococcus (OMPC). Proteins such as diphtheria and tetanus toxoids, which derive from the respective toxins after chemical detoxification with formaldehyde, were initially selected as carriers because of their safety record established over decades of vaccination against tetanus and diphtheria. CRM197, a 58 kDa nontoxic mutant of diphtheria toxin, isolated from the supernatant of cultures of *Corynebacterium diphtheriae* C7(β197) tox(-) strain (Giannini et al. 1984), has the advantage of not requiring chemical detoxification. CRM197 has been used extensively as a carrier for licensed Hib and multivalent meningococcal and pneumococcal conjugate vaccines and other vaccines in development (Bröker et al. 2011; Shinefield 2010). OMPC has been used for a Hib conjugate vaccine (Donnelly et al. 1990) and a first generation of pneumococcal conjugate vaccines (Kilpi et al. 2003), while protein D was introduced as a carrier for most of the serotypes in a multivalent pneumococcal conjugate vaccine (Forsgren and Riesbeck 2008; Prymula et al. 2006). Protein D (PD) is a 40 kDa cell-surface protein originally derived from non-typeable *H. influenzae* and produced from a recombinant strain of *E. coli.*

8.4.2 Characterization of Protein Carriers

Characterization of the carrier protein is required as part of the product-licensing portfolio. When tetanus and diphtheria toxoid are used in glycoconjugate vaccine manufacture, they should at least meet the WHO and Pharmacopoeial requirements established for the corresponding 'stand-alone' vaccine. The key quality parameters for toxoids are those defined by the release tests performed on the concentrated bulk. The purity (and identity) of toxoids can be measured using methods ranging from immunochemical to physicochemical. The immunochemical methods make use of reference sera and can be immunoprecipitation (the classical flocculation determination), immunoelectrophoretic or immunoenzymatic methods, whereas physicochemical techniques includes SDS gel electrophoresis, isoelectric focusing and SEC-HPLC. In addition, the proportion of monomer and multimeric forms of the toxoid needs to be evaluated; some manufacturers perform further purification of toxoids lots if used for conjugate production. Alternatively, toxin purification

may precede detoxification since this may result in a purer product, but particular care must be taken to avoid reversion to toxin when this procedure is used (Frech et al. 2000). Further detailed characterization of toxoids is typically not performed as part of conjugate development, although techniques applicable to achieve this have been described for use in controlling the detoxification process and monitoring tetanus toxoid vaccine production (Metz et al. 2013).

The third carrier OMPC is extracted from washed bacterial cells with buffer containing detergent and further processed to remove cell debris impurities including lipopolysaccharide (LPS). The protein composition can be profiled by SDS-PAGE or similar methods and the LPS should not exceed 8 % by weight (Anonymous 2000). The OMPC lot must pass the rabbit pyrogen test at 0.25 μg/kg of body mass and the LPS content measured. HPLC (Li et al. 2004; Lyngby et al. 2002) and GC-MS (de Santana-Filho et al. 2012) methods have been developed for LPS quantitation; they are based on fatty acid content and are more specific and sensitive than colorimetric or SDS-PAGE methods previously used. Advances in bioanalytical characterization of such products have been facilitated by the recent licensure of a multicomponent vaccine against group meningococcal B disease, which includes outer membrane vesicles (Gorringe and Pajon 2012; Ravenscroft et al. 2010; Vipond et al. 2006).

Key quality attributes for those carrier proteins that are not stand alone vaccines, such as CRM197 and PD include: structure/identity, sterility or bioburden (depending on the manufacturing process), endotoxins, and purity. In some specific cases, additional quality attributes may require measurement. Although immunochemical methods are applied, characterization of the concentrated bulk is mainly performed by use of physicochemical methods including SDS-PAGE, isoelectric focusing, HPLC, amino acid analysis, amino acid sequencing, peptide mapping, and mass spectrometry (Hsieh 2000). Even though the conformation of the carrier protein may not directly determine the immunogenicity of the glycoconjugate vaccine, determination of secondary and tertiary structure is helpful in assessing batch to batch consistency and might be useful as part of comparability studies when changes of process or manufacturing sites occur. Techniques including far and near UV circular dichroism, fluorescence spectroscopy, and differential scanning calorimetry (DSC) have been applied (Crane et al. 1997; Ho et al. 2000, 2001, 2002). CRM197 is a well-established carrier protein used in several currently licensed conjugate vaccines (Bröker et al. 2011; Shinefield 2010); it has been fully characterized and the structural basis for its lack of toxicity compared to diphtheria toxin recently explained (Malito et al. 2012). The quality criteria and testing established during its licensure thus serve as the model for release of new carrier proteins. Where CRM197 is produced in the same facility as diphtheria toxin, proof of non-toxicity is required. Typical expectations are that the purity of CRM197 should be at least 90 % and often >95 % (with dimers and higher oligomers <5 %). CRM197 contains an exposed loop of three arginines, which is clipped by proteases present in the culture medium resulting in a so-called nicked form. The degree of nicking needs to be controlled (typically <5 % of total CRM197); the proportion of intact CRM and fragments A and B can be readily determined by SDS-PAGE

performed under reducing conditions. Protein carriers obtained with recombinant DNA technology, such as PD (and CRM197 in development), need to comply with additional requirements established for recombinant products including testing for host cell DNA and proteins (Brady et al. 2012).

8.4.3 Characterization of Derivatized Protein Carriers

Most conjugation methods make use of the carboxyl groups of glutamic and aspartic acids or the primary amines (lysine residues and the N-terminal) or thiols of the protein carrier. However, some strategies require prior derivatization of the protein carrier to generate functional groups that will react specifically with active groups on the saccharide intermediate. The issues discussed for saccharide intermediates apply, namely, that the extent of derivatization must be quantified and that "over-derivatization" should be avoided as it might mask potential T cell epitopes of the carrier protein (Pon 2012). In general, such functional groups are introduced by derivatization of protein amino acids side chains such as glutamic/aspartic acid with a bifunctional reagent (e.g., adipic acid dihydrazide or hydrazine) so that a highly nucleophilic hydrazide group becomes available for coupling with the polysaccharide (Schafer et al. 2000). This approach is also applicable to toxoids and was used by the Meningitis Vaccine Project to improve the coupling of periodate-activated meningococcal group A polysaccharide to TT (Lee et al. 2009) and other groups for conjugates in development against Hib (Laferriere et al. 2011) and meningococcal group C disease (Silveira et al. 2007). In other manufacturing strategies, the lysine side chains of the carrier protein can be derivatized to introduce different reactive groups (e.g., bromoacyl, thiol groups or maleimido groups). The Hib-OMPC conjugate is prepared using this technology, the bromoacetylated activated Hib polysaccharide (Table 8.4) is coupled to the thiolated protein prepared by reaction of OMPC with *N*-acetylhomocysteine thiolactone (Marburg et al. 1986). Calculation of the extent of protein derivatization requires appropriate methods for determination of the amount of functional groups introduced and measurement of total protein. The extent of hydrazide-derivatization of proteins can be determined using colorimetric assays (Qi et al. 1988), whereas the introduction of thiols is measured by a reaction of Ellman's reagent with free thiols (Ellman 1959). Methods used for total quantity of protein include amino acid analysis, spectrophotometric and colorimetric assays or other appropriate methods; there should be no interference from the functional group introduced. Alternatively, mass spectrometry can be applied: MALDI-TOF MS analysis of ADH-derivatized CRM197 showed a mass increase of 830 Da compared with underivatized CRM197 corresponding to the attachment of approximately 5 ADH linkers per CRM197 (Micoli et al. 2011a). In some cases, the procedures for protein carrier activation or derivatization may result in a certain degree of covalent aggregation of the carrier itself, and this should be monitored with appropriate tests like SEC-HPLC coupled to static light scattering detection, SDS-PAGE, mass spectrometry, or other suitable tests.

8.5 The Glycoconjugate Vaccine (Drug Substance)

The glycoconjugate vaccines formed from conjugation of randomly-activated polysaccharides or terminally-activated saccharides to carrier proteins are complex and consist of a family of glycoforms for which only the average properties can be readily measured. Therefore, strict control of the quality of the activated saccharide and carrier protein, as well as the conjugation conditions are required to ensure consistent production of these structurally diverse biologicals. Both the multistep methods and the control procedures used to ensure the reproducibility, stability, and safety of the conjugate should be established once the immunogenicity of the conjugate vaccine has been demonstrated. The success of the chemical coupling of a specific activated saccharide and protein depends on many variables including the relative concentrations (and sizes) of the reactants, coupling chemistry/reactive groups, uniform mixing, temperature, pH, and ionic strength, all of which need to be carefully controlled to maximize the yield and minimize cross-linking and precipitation. Ways to improve conjugation efficiency include size-reduction of native polysaccharides, particularly if they are viscous, and the use of spacers or linkers that help to overcome electrostatic and steric barriers to linkage formation between the saccharide and protein macromolecules. Mild conjugation conditions that have little impact on the structural integrity of the saccharide or protein and do not introduce new epitopes (neoepitopes) are desirable. Less control is possible for randomly-activated polysaccharides that form multiple points of attachment with the carrier protein; the saccharide protein ratio of the conjugate formed depends on the input masses, while the conjugation time required depends on the conjugation chemistry and can be followed by SEC-HPLC. Conjugations using cyanylation chemistry are fast: for example CDAP activation may occur over several minutes and the conjugation reaction is quenched after 1 h by the addition of glycine (Capiau et al. 2008); whereas reductive amination coupling is slow and may take from 8 h to several days depending on the polysaccharide structure, quenching is by sodium borohydride reduction of the saccharide aldehydes (Prasad 2011). For monomeric conjugates, the saccharide/protein ratio and the size can be controlled and to some extent defined, firstly by the size distribution of the oligosaccharides selected for terminal active ester derivatization and secondly by the ratio of active ester to protein used for the conjugation reaction. The conjugation reaction is terminated either by removal of the unreacted reactants (e.g., by diafiltration) or by chemical deactivation/quenching or capping of residual reactive groups before purification of the conjugate from conjugation reagents and unconjugated saccharide and protein. The techniques employed are vaccine-specific and generally rely on the higher molecular weight (and larger hydrodynamic size) or different charge or hydrophilicity of the conjugate formed compared to the unconjugated saccharide and protein reactants. Methods used include diafiltration, size exclusion chromatography, selective precipitation (e.g., ammonium sulfate), hydrophobic interaction chromatography (HIC) or combinations of these techniques (McMaster 2000).

After the bulk conjugate has been purified, the tests listed in Fig. 8.2 should be performed to assess consistency of production. The tests are critical for assuring lot-to-lot consistency and are described in the WHO and Pharmacopoeia guidelines. Most of the control testing of the conjugate vaccine is vaccine-specific and includes purity (free from residual reagents), the absence of activated functional groups (used for conjugation) and specific toxicity of the carrier protein (when toxoids have been used). Proof of the covalent linkage (an essential feature of conjugate vaccines) between the saccharide and carrier protein must be established, and its extent determined. The structure of the conjugate must be characterized with respect to the molecular size distribution, the saccharide and protein content, and the ratio of saccharide to protein calculated. The amount of free (unbound) saccharide present in the vaccine must be determined; this serves as a key stability indicator for conjugate vaccines. The physicochemical procedures used for analysis and control of conjugate vaccines are based on the methods already described for analysis of the carrier protein and the activated saccharide intermediate; their application can reveal structural changes arising from the conjugation reaction such as unfolding of the protein carrier or changes in the structure of the saccharide chain caused by cleavage or loss of labile substituents. The level of physicochemical characterization possible varies with the type of conjugate: the simple monomeric type of conjugate (iii) with CRM197 as carrier is the most amenable to structural characterization, whereas a cross-linked lattice conjugate (i) formed with a toxoid as carrier, presents the biggest challenge to structural characterization (Bardotti et al. 2008; Bolgiano et al. 2001; Ho et al. 2000; Jumel et al. 2002). Nevertheless, the chromatographic and spectroscopic data spectra obtained throughout the process constitute structural fingerprints of the vaccine intermediates and final product and provide a sensitive probe of manufacturing consistency. These data can also be used to support proof of bioequivalence between vaccine lots when changes in the manufacturing process have been implemented. A summary of the physicochemical methods applied to the characterization of glycoconjugate vaccines is shown in Fig. 8.7.

8.5.1 Proof of Conjugation

An essential feature of conjugate vaccines is the formation of a covalent linkage between the saccharide and carrier protein. Proof of conjugation may be demonstrated by co-elution of the saccharide and protein components on SDS-PAGE, size exclusion chromatography or capillary electrophoresis; a physical mixture of the protein and saccharide should be used as a control. Conjugation alters the migration of the carrier protein in SDS-PAGE resulting in a broad smear of higher molecular weight due to the conjugate; co-migration of the saccharide is shown by staining or Western blot. Similarly, size exclusion chromatography with UV/RI detection can profile the formation of the conjugate of increased hydrodynamic size compared to the saccharide and protein reactants; co-elution can be confirmed by fraction

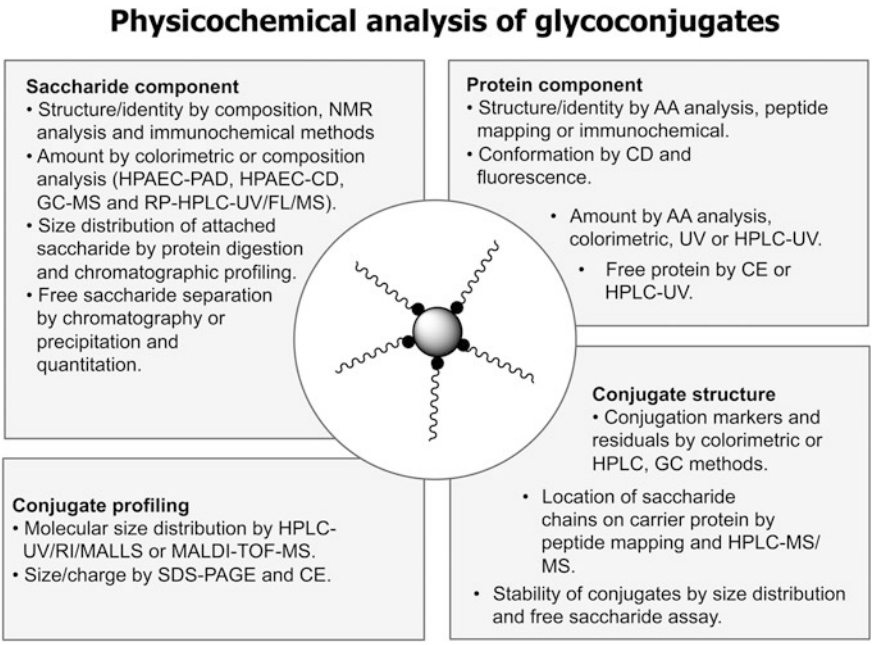


Fig. 8.7 A summary of the physicochemical methods applied to the characterization of glycoconjugate vaccines

collection and testing using spectrophotometric, chemical, or immunochemical methods. A column with an appropriate fractionation range should be selected as the high molecular weight "conjugate" eluting in the void volume of the column may simply be a noncovalent mixture of the polysaccharide and aggregated protein carrier. The applicability of these techniques depend on the nature of the conjugate formed; some high molecular weight cross-linked lattice conjugates (Fig. 8.1, approach (i)) may be too large to enter the gel or column, although their formation can be demonstrated by size analysis using static light scattering, such conjugates may be lost during downstream processing. On the other hand, only small changes in migration and hydrodynamic size would be observed for monomeric conjugates with a low loading of neutral saccharides; however, such conjugates are amenable to analysis by mass spectrometry (Kabanova et al. 2010a, b; Oberli et al. 2011). Some methods of conjugation result in the formation of stable covalent bonds that are not cleaved by the hydrolysis conditions employed for amino acid hydrolysis. The modified amino acid formed can be quantified and serves as a unique marker for the degree of conjugation. Saccharide aldehydes that are reductively aminated to the carrier protein form a modified lysine residue, which can be detected by amino acid analysis; typically 6–9 of the 39 lysines are conjugated (Seid et al. 1989; Hsieh 2000). Similarly, the bromoacetylated saccharide coupling to the thiolated protein yields an S-carboxymethyl homocysteine residue (Marburg et al. 1986). Most of the

other coupling chemistry listed in Table 8.4 yields saccharide or linker-protein linkages that are cleaved during the conditions required for amino acid hydrolysis, and therefore the degree of conjugation is inferred from the migration or elution of the conjugate formed. Alternatively, if the conjugate can be deglycosylated by acid hydrolysis conditions that are mild enough to leave the protein carrier intact with a sugar fragment linked directly or via a spacer to the amino acid involved in the covalent linkage (an adduct, Bardotti et al. 2008), then the average number of attachment sites per protein can be determined by MALDI-TOF-MS.

Capture ELISA is an alternative approach not requiring chromatography or detection of a conjugation marker. In the first step, the plate is coated with saccharide antibody to capture all the saccharide present (free and conjugated), after washing of unbound material, protein carrier antibody is used to detect the protein component of the conjugate. Lastly, indirect evidence of conjugation for some conjugates may be obtained through animal studies that elicit a strong immune response to conjugates compared to the corresponding polysaccharide alone (Suker et al. 2004).

8.5.2 Saccharide Identity and Content

Immunological or chemical tests are performed on the monovalent bulk to confirm the saccharide identity and demonstrate that the multistep conjugation process did not significantly alter or destroy important epitopes. The tests employed are based on those described for establishing polysaccharide structure and identity (Sect. 8.2.2) and for confirming the structural integrity of the activated intermediate (Sect. 8.3.2), however, for conjugates there might be interference from the carrier protein. Immunological assays thus require appropriate reagents to ensure that they are specific for the antigen concerned; methods applied include ELISA, immunoblot analysis, and rate nephelometry. Alternatively, the structural identity of the saccharide antigen can be confirmed using physicochemical methods such as colorimetric (for simple repeating units) or composition analysis (Table 8.3) if acceptable specificity can be demonstrated. Once again, NMR spectroscopy is the preferred physicochemical technique in terms of specificity and the structure-rich information provided on saccharide sugar composition and substituents. The NMR spectra of conjugates contain relatively sharp signals from the saccharide component superimposed on broad peaks of low intensity from the carrier protein (Fig. 8.8), and therefore the saccharide structures of all currently licensed conjugate vaccines (Hib, 4 meningococcal and 13 pneumococcal) can be identified. The technique is applicable to both polysaccharide and oligosaccharide-based conjugates, but depends on the saccharide loading and the nature of the protein carrier. Monomeric CRM197 conjugates are the most amenable to NMR analysis, whereas cross-linked Hib conjugate vaccines with tetanus toxoid or OMPC as carriers do not give acceptable spectra (Aubin et al. 2010; Jones and Ravenscroft 2008). As for the polysaccharide identity test, the spectra of *O*-acetylated antigens can be simplified by in situ base treatment.

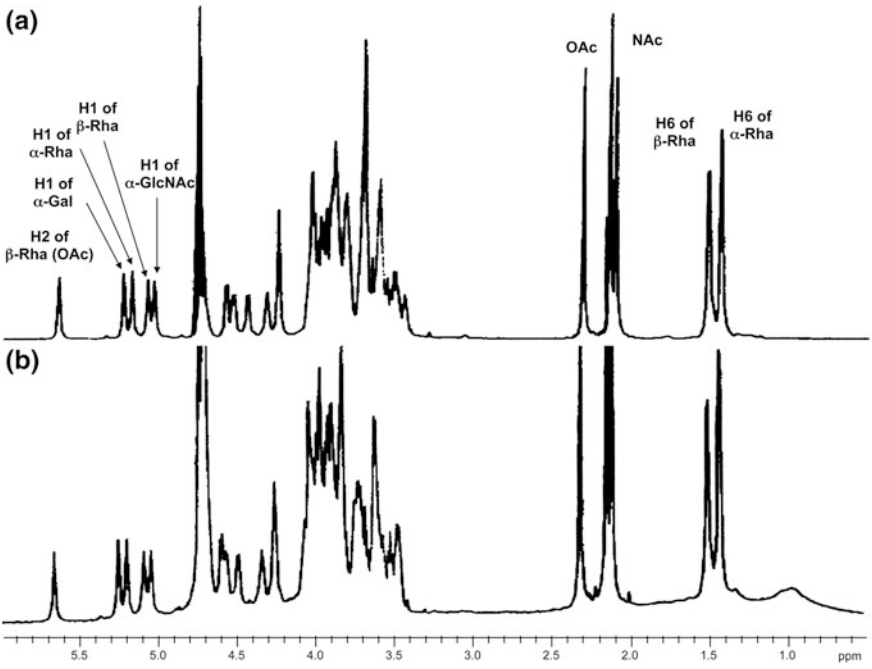


Fig. 8.8 ^{1}H NMR spectra of pneumococcal serotype 7F polysaccharide (**a**) and the corresponding tetanus toxoid conjugate (**b**); some diagnostic resonances are labeled. See Table 8.2 for the structure of the Pn 7F polysaccharide repeating unit

The saccharide content of the conjugate must be determined; this quantity is used to characterize the conjugate, calculate the yield and recovery of the conjugation reaction, and importantly define the dose of the antigen in the final drug product. In principle, the methods available include colorimetric and composition assays (Table 8.3) or immunological methods such as ELISA or rate nephelometry (if reagents are available). In practice, the same assay used to determine the saccharide input for the conjugation reaction is also applied to determine the saccharide content of the conjugate. These are typically appropriate colorimetric assays for specific components (ribose, phosphate, sialic acid, or uronic acid) or the general carbohydrate assays (phenol/sulfuric or anthrone). The assay should be specific and not suffer from interference from the carrier protein. The informative composition assays based on depolymerisation and monomer quantification by use of HPLC, HPAEC-PAD, or GC are largely used during optimization of the activation/conjugation conditions and process development, but not for the saccharide content release assay. By establishing a correlation between the colorimetric and immunochemical assays (ELISA and RN) for the monovalent bulk, immunochemical assays can be used for saccharide antigen content in the drug product (Sect. 8.6), where low amounts and multivalent formulations preclude the use of chemical assays.

8.5.3 Protein Identity and Content

The identity of the carrier protein may be confirmed by use of an immunological method such as ELISA. This identity test, or a more detailed structural characterization of the carrier protein, would only be considered if multiple protein carriers were used within the same manufacturing facility. The protein content of the conjugate must be determined; this quantity is used to characterize the conjugate and monitor the consistency of the conjugation reaction. It will also allow determination of the protein content in the final drug product. Methods used for total quantity of protein include amino acid analysis, spectrophotometric (UV absorbance), and colorimetric assays [Lowry et al. (1951), bicinchoninic acid assay, Smith et al. (1985) or Bradford (1976)]; the method chosen must not suffer from interference from the saccharide component or residual functional groups that may be present from the conjugation. Typically, the method used for the conjugate is the same as the method used to determine the protein input for the conjugation reaction (Sect. 8.4.3).

8.5.4 Saccharide–Protein Ratio

The saccharide–protein ratio for a conjugate, also known as the degree of glycosylation, is calculated from the ratio of concentrations (mass/volume) for the saccharide and protein content, respectively. The concentrations are typically measured using two independent assays as described in Sects. 8.5.2 and 8.5.3. This ratio reflects the relative proportions of the saccharide and protein that reacted to form the conjugate, and is thus also an indicator of process consistency. The value reported should be in the range approved for that conjugate and should be consistent with vaccine shown to be effective in clinical trials. For vaccines licensed to date, Hib vaccines contain the largest variation in ratio ranging from <0.1 (Hib-OMPC) to 1.4 (Hib-DT), whereas the meningococcal MCV4 vaccines contain average ratios of 0.33 (DT), 0.45 (TT), and 0.52 (CRM197). For pneumococcal conjugate vaccines, the ratio is typically in the range 0.3–3.0 depending on the serotype (Anonymous 2005), with average ratios of 0.80 (PCV7), 0.65 (PCV10), and 0.96 (PCV13) calculated from the respective product compositions listed.

In principle, several physicochemical methods can give a direct measure of the saccharide–protein ratio, although they are not necessarily used for lot release. SEC-HPLC-UV/RI with dual monitoring (e.g., refractive index and UV, for total material and protein content, respectively) can profile the saccharide/protein ratio over the size distribution of conjugates, which provides a more sensitive probe of manufacturing consistency that the average value from the saccharide–protein content (Hsieh 2000). Conjugates that are suitable for proton NMR analysis (Sect. 8.5.2), yield spectra containing sharp diagnostic signals for the saccharide component and broad signals for the folded carrier protein. However, if the protein

is denatured by addition of guanidium hydrochloride or base, then the resonances from the aromatic amino acid sidechains provide a measure of the protein content, and one of more of the sugar-derived resonances can be integrated to determine the saccharide content (Jones and Ravenscroft 2008). The molecular mass of well-defined monomeric conjugates can be measured by SEC-HPLC-MALLS or MALDI-TOF-MS and by knowing the protein component, the attached saccharide mass determined.

8.5.5 Molecular Size Distribution

The molecular size of the conjugate is a key indicator of process consistency and provides proof of conjugation. Size analysis also indicates the integrity of the conjugate particularly if the saccharide component is susceptible to hydrolysis, and is used as a valuable stability-indicating assay. The method used is vaccine-specific and includes size exclusion chromatography, SEC-HPLC-UV/RI/MALLS, analytical ultracentrifugation, or dynamic light scattering. Historically, size analysis criteria for conjugates were based on the methods established for the polysaccharide vaccines, although unlike polysaccharide vaccines, the molecular size of the conjugate does not appear to relate to clinical efficacy. The specifications were based on K_D values obtained on specified columns with peak analysis by UV, RI, colorimetric assay, or ELISA. Typically SEC-HPLC-UV/RI is used to track the conjugation from starting polysaccharide and protein, through the intermediates to the final conjugate. A single column (or two in series) may be used with calibration using molecular weight standards and the peak apparent molecular weight and size distribution reported. Conjugates that display several major peaks may require additional description. The recovery of the conjugate through the column should be demonstrated as large cross-linked conjugates and aggregates will not be eluted although they are present and contribute to the other conjugate parameters measured. Use of the MALLS detector provides a more quantitative estimate of size and polydispersity; analysis of Mn group C vaccines shows that the conjugates prepared using periodate activation (approach (ii), Fig. 8.1) are cross-linked to several protein carriers, whereas the oligosaccharide terminally activated conjugates (approach (iii), Fig. 8.1) are monomeric (Bardotti et al. 2008; Jumel et al. 2002).

8.5.6 Free Saccharide Assay

The term free saccharide refers to the amount or proportion of saccharide that is present but not covalently linked to the protein carrier. It may be a process residual remaining after purification of the conjugate, i.e., unconjugated saccharide, or a product related impurity generated by hydrolysis of the glycoconjugate. This parameter is important because only saccharide that is covalently bound to the

carrier protein, i.e., conjugated saccharide is immunologically important for clinical protection, and secondly, the presence of large amounts of free saccharide may suppress the immune response to the antigen (Peeters et al. 1992; Poolman and Borrow 2011; Rodriguez et al. 1998). Therefore, each batch of conjugate should be tested for unbound or free saccharide in order to ensure that the amount present in the purified bulk at release and during its storage is within the limits for lots shown to be clinically safe and efficacious. As a guideline, two of the first generation of licensed conjugates had free saccharide specifications of <20 % (Hib-TT) and <25 % (Hib-CRM197), respectively (Anonymous 2008); lower levels are expected today. The stability of glycoconjugates based on free saccharide content varies depending on the type of conjugate and structure of the saccharide repeating unit (and the nature of glycosidic bonds present). Cross-linked lattice conjugates (Fig. 8.1, approach (i)) have multiple saccharide–protein linkages, and therefore a cleavage in the saccharide chain may not release free saccharide, whereas it would be detectable for monomeric conjugates (Fig. 8.1, approach (iii)). Apart from minor differences due to conjugate architecture, the main determinant for conjugate stability is the inherent stability of the saccharide chain under the conditions of storage; this is discussed further under stability testing (Sect. 8.5.11). Analytical tests to monitor the free saccharide are of paramount importance since this parameter is considered an indicator of vaccine potency and stability, and is a critical factor used to establish the expiry date for the product.

Determination of the level of free (or unconjugated) saccharide first requires that it be separated from the conjugated saccharide. Methods that have been used include chromatography (size, ion-exchange or hydrophobic interaction), chemical precipitation of the conjugate with acid and/or detergents, ammonium sulfate or by aluminum adsorption, capillary electrophoresis, gel filtration, centrifugal ultrafiltration, ultracentrifugation, solid phase extraction, or immunoprecipitation. The amount of free saccharide can then be quantitated using the method that was used to quantitate the total saccharide content (if that method is sufficiently sensitive), which is by specific chemical or immunological assays, or by composition analysis using HPLC, HPAEC, or GC after hydrolysis. Finally, the level of free saccharide is related to the total saccharide by calculating the ratio between the respective concentrations to provide an assessment of the effectiveness of the conjugate purification process and integrity of the glycoconjugate. The methods employed for the separation and subsequent free saccharide quantification depend on the nature of the conjugate and the saccharide component. A key challenge to the physical separation of conjugate and free saccharide are the electrostatic interactions between them and the relatively large hydrodynamic size of saccharides, and therefore the method chosen has to be carefully validated and include evaluation of samples spiked with protein carrier as well as samples spiked with activated saccharide to ensure robustness of the free saccharide assay. Use of forced degradation studies during vaccine development (Sect. 8.5.10) can be used to demonstrate the suitability of the method chosen. Analysis of the large Hib-OMPC conjugate uses ultracentrifugation (Belfast et al. 2006), whereas the deoxycholate/HCl precipitation method seems to be generally applicable to Hib (Guo et al. 1998; Lei et al. 2000a), meningococcal

(Lei et al. 2000b) and pneumococcal conjugates. Centrifugal ultrafiltration with the appropriate membrane cut-off has been applied to Hib and polysaccharide and oligosaccharide-based conjugates (Bolgiano et al. 2007) as well as meningococcal oligosaccharide-CRM197 conjugates (Bardotti et al. 2008). Solid phase extraction (SPE) is widely used for sample preparation in drug analysis, and has been applied to retain the intact Hib and meningococcal oligosaccharide-CRM197 conjugate on C4 cartridges with elution and analysis of the corresponding free saccharide fraction by HPAEC (Carinci et al. 2012).

The application of CE to the analysis of meningococcal polysaccharides (Sect. 8.2.2) and saccharide derivatives (Sect. 8.3.2) has been extended to the corresponding diphtheria toxoid conjugates. Micellar electrokinetic chromatography (MEKC) was found to provide separation of the meningococcal (A, C, Y, and W) conjugates and free DT and with use of an internal standard (myoglobin) used to quantify unconjugated DT. The technique was also shown to be applicable to several pneumococcal polysaccharides and their corresponding TT and DT conjugates (Lamb et al. 2000). The success of the separations was polysaccharide-specific, but suggests that CE has the potential to quantify conjugate, free saccharide, and free protein in a single experiment. Similarly, CZE was able to resolve and quantify the meningococcal (A, C, Y, and W) CRM197 conjugates (Carinci et al. 2012).

8.5.7 Unconjugated Carrier Protein

As for free saccharide, the level of unconjugated (or free) carrier should be monitored for each lot of monovalent conjugate because it is a key maker of process consistency, an indirect measure of covalency and could interfere with the immune response against the glycoconjugate. The unconjugated protein can be separated from the conjugate and simultaneously quantitatively determined by use of SEC-HPLC using UV or fluorescence detection, SDS-PAGE followed by staining and scanning of the gel or by capillary electrophoresis (Lamb et al. 2000). In all cases, the calibration curve is established with a reference unconjugated protein (or derivatized protein) and validated by use of spike recovery experiments.

8.5.8 Unreacted Functional Groups and Capping Markers

Residual unreacted functional groups capable of reacting in vivo may be present on the activated saccharide and/or derivatized protein after the conjugation process. The nature of the functional group depends on the coupling chemistry (Table 8.4), and if considered reactive, may be chemically quenched or reacted with a small molecule to cap the active group. Additionally, the presence of residual reactive groups may affect product stability during storage. Residual cyanoesters and imidocarbonates resulting from CNBr (or CDAP) activation of the saccharide are

deactivated by the addition of simple amines such as glycine or ethanolamine; the amount of the cap introduced can be monitored by hydrolysis and chromatographic methods (HPLC, GC, or amino acid analysis) or NMR spectroscopy if appropriate. Reactive bromoacetamide residues on the saccharide are capped with *N*-acetylcysteamine, which is detected as the unique amino acid S-carboxymethyl cysteamine by amino acid analysis. Thus, amino acid hydrolysis allows quantification of the total degree of protein activation from the sum of the protein active sites involved in conjugation (as *S*-carboxymethylhomocysteine) and the sites activated but not conjugated (as *S*-carboxymethyl cysteamine); this serves as an analytical check for overderivatization and possible reduction of potential T-cell epitopes (Kniskern and Marburg 1994). Reactive aldehydes remaining after reductive amination are typically removed by reduction with sodium borohydride to form an unreactive alcohol; this reduction step also "locks" the imine bonds formed between the protein amine and saccharide aldehyde. Detection of low amounts of unreduced aldehydes remaining after quenching presents an analytical challenge due to the protein present; one strategy would be to selectively react the aldehyde with a hydrazide- or aminooxy-functionalized fluorophore, which would be linked to the conjugate and detectable by SEC-HPLC-FL (Johnson and Spence 2010). Unreacted hydrazides present on the randomly activated saccharide or derivatized protein are not considered to be reactive, but should be monitored by use of the selective colorimetric or chromatographic methods described in Sect. 8.3.2. The active groups present at the terminals of saccharides (active ester or hydrazide) are not a concern if they remain unreacted during conjugation as they will be removed together with the unconjugated saccharide during conjugate purification. In general, each batch should be shown to be free of unreacted functional groups; alternatively the product of the capping reaction can be monitored. Validation of the manufacturing process for removal of unreacted functional groups by quenching or capping during vaccine development can eliminate the need to perform this analysis for routine control.

8.5.9 Residual Reagents

The conjugate purification procedures should remove residual reagents used for conjugation and capping. The nature of the reagents and reaction by-products (and their potential toxicity) depend on the conjugation chemistry employed (Table 8.4) and their removal should be confirmed by suitable tests or by validation of the purification process. Appropriate methods may include colorimetric, hyphenated chromatographic techniques and NMR spectroscopy. Cyanylation chemistry and reductive amination require analysis of cyanide (colorimetric or ion chromatography) and the use of CDAP releases DMAP (4-dimethylamino-pyridine), which must be monitored. Several methods have been reported for the analysis of residuals from conjugations using carboxyl groups and spacers including a comprehensive single method employing RP-HPLC-MS/MS for the carbodiimide, by-products and a diamine spacer (Lei et al. 2004); the spacer can also be quantified using

fluorescent derivatization and RP-HPLC or CZE as described in Sect. 8.3.2 (Adam and Moreau 2000). Lastly determination of hydrazine in the presence of protein was achieved using differential pulse polarography instead of colorimetric assays (Bastos et al. 2010). Consistency in the amount of residual reagents from the conjugation chemistry can be demonstrated during process development, and the process can be validated for their clearance. This validation includes not only unconjugated polysaccharide and protein, but also buffers, salts, small-molecule reaction components, and by-products generated during conjugation. Provided that consistent levels of residual solvents are recovered, such testing may serve as an inprocess control.

8.5.10 Conjugate Structure

For some conjugates, additional detailed structural characterization may be performed based on the technologies developed for the analysis of "natural" glycoproteins: glycomics and proteomics. Unfortunately, many of the specific enzymes used for release of *N*- and *O*-linked glycans and sequential deglycosylation steps for sugar chains are not applicable to current thiol, carboxyl, or amino-linked glycoconjugate vaccines. Some of these techniques have been used for the characterization of CRM197 conjugates (Hsieh 2000; Jones et al. 2000) with the "state-of-the-art" described by Bardotti et al. (2008) in their study of a tetravalent meningococcal conjugate vaccine. Both the periodate oxidation and active ester conjugations to CRM197 are through nucleophilic attack by the ε-amino groups of Lys residues and the N-terminus. Progress has been made in determining the average number of linkage sites (Sect. 8.5.1) and the next step is to identify their location and establish whether certain sites are preferentially glycosylated. For active ester chemistry, the reactivity will depend of the amino group's pKa and steric factors, whereas reductive amination may be more complex owing to the formation of an intermediate Schiff's base (which is subsequently reduced). Most of the published studies have examined products prepared from the active ester conjugation coupling to CRM197. Peptide mapping studies performed by Jones et al. (2000) confirmed the sequence of CRM197, and the HPLC-ES-MS profile showed that Asn-N digestion was more effective than the use of Glu-C or trypsin. Fluorescent labeling of CRM197 using the same conjugation chemistry and peptide mapping with fluorescence detection suggested that there were about eight preferred glycosylation sites. A well-defined conjugate consisting of a single repeating unit of Pn 19F conjugated to CRM197 (13 chains per protein) was subjected to peptide mapping. Asn-N digestion and HPLC-ES-MS identified seven major glycosylation sites with a high degree of certainty and several others, which can be located only to a particular Asn-N peptide containing multiple Lys residues. The seven sites could be mapped onto the crystal structure of diphtheria toxin and all are located on the protein surface and available for conjugation.

For licensed vaccines, location of the saccharide chains on the protein by LC-MS of the peptides and glycopeptides produced by proteolytic digestion is complicated by the structural heterogeneity of the saccharide chains. This was overcome by Bardotti et al. (2008) by modifying the peptide mapping procedure to include a deglycosylation step; this approach was applied to analysis of a tetravalent meningococcal conjugate vaccine. Following tryptic digestion, the oligosaccharide-bearing peptides (of relatively large hydrodynamic size compared to peptides) were isolated using SEC-LC-MS. The glycopeptides were deglycosylated by acid treatment to form the peptide-adducts, which were identified by RP-LC-MS with MS/MS performed on some glycopeptides. The LC-MS technique is not quantitative; however, between 29 and 34 of the 39 lysines were involved in conjugation, with 19 glycosylated in all lots examined. This demonstrates consistency in the active ester-CRM197 conjugation irrespective of the saccharide employed, while the large number of lysine residues that are modified suggests that the conjugation is not highly specific in nature.

As part of this study, NMR analysis (of the saccharide) and detailed size analysis of the conjugate was reported, and the conformation of the protein carrier investigated. Optical spectroscopy using circular dichroism (CD) and fluorescence spectroscopy were applied to CRM197 before and after conjugation. Predictions from the far-UV CD spectra showed that conjugation did not induce significant conformational changes to CRM197, whereas a more open conformation was indicated by the fluorescence data. These results are in agreement with previous studies performed on Hib and meningococcal group C CRM197 conjugates (Crane et al. 1997; Ho et al. 2001). The estimations of secondary structure can be used to demonstrate lot-to-lot consistency; however, the importance of preservation of the "native" folding of the carrier protein to immunogenicity of glycoconjugate vaccines has not been demonstrated (Ho et al. 2000).

8.5.11 Stability Testing

The stability of the monovalent bulk conjugate needs to be evaluated as part of vaccine development and appropriate testing methods established. Hasija et al. (2013) have reviewed the mechanisms by which vaccines degrade (chemical and physical), the analytical tools available and provided examples of forced degradation studies, a summary of vaccine potential stabilizers and a list of the ICH (International Conference on Harmonization) guidelines on stability studies. The primary structure of proteins can be altered by deamidation, oxidation, disulfide exchange, or non-enzymic cleavage, whereas alteration of secondary, tertiary, or quaternary structure can alter key epitopes or expose nonpolar regions and result in aggregation or precipitation. Saccharides are subject to cleavage and the loss or migration of labile groups such as *O*-acetyls. Based on extensive experience with Hib and monovalent meningococcal group C conjugates, the protein carrier appears to be stabilized by the glycosylation, the covalent linkages between the protein and

saccharide (or via a linker) are generally stable, and therefore the "weakest link" is the saccharide itself (Hsieh 2000). The saccharide component of conjugate vaccines may be subject to gradual hydrolysis at a rate, which may vary depending upon the type of conjugate, the type of formulation (pH, ionic strength, buffer and excipients) and conditions of storage. The hydrolysis may result in reduced molecular size of the saccharide component, a reduction in the amount of the saccharide bound to the protein carrier and in a reduced molecular size of the conjugate and a decrease in vaccine potency. The immunogenicity may also be affected by the novel saccharide chain end groups generated by depolymerization (neoepitopes, Egan 2000) and the increase in the amount of free saccharide (Sect. 8.5.6). Consequently, the key physicochemical characteristics indicating stability have been identified as free saccharide, molecular size, and antigenicity (Hsieh 2000).

The degradation of conjugates under normal storage conditions is slow, and therefore the development of appropriate physicochemical (and animal) tests to properly assess the integrity of conjugate vaccines follows from accelerated stability studies otherwise known as forced degradation studies (Hasija et al. 2013). Following the expertise and understanding gained from studies of Hib and meningococcal group C CRM197 and TT conjugates (Crane et al. 1997; Ho et al. 2000, 2001, 2002), stability studies conducted at different temperatures and by freeze-thaw experiments not only show which tests are useful, but also predict the relative stability and the shelf life of the bulk and formulated vaccines. The structural stability, hydrodynamic size, and molecular integrity of the treated vaccines were monitored by optical and NMR spectroscopy, size exclusion chromatography, and free saccharide (by use of HPAEC-PAD). The results showed that all the conjugate vaccines were stable when stored at the recommended temperatures, but differences in the structural stability (and immunogenicity) of the conjugates were observed at elevated temperatures and after cycles of freeze-thawing; these were attributed to differences in conjugate structure, the coupling chemistry employed, protein carrier, and formulation. The studies confirmed that saccharide integrity, as indicated by the free saccharide and size distribution assays, determines the immunogenicity of conjugate vaccines and that it is not affected by protein conformation. However, the sole use of free saccharide as a measure of structural integrity for different types of conjugates is not straightforward; for example, a single cleavage in the saccharide chain of a monomeric type of conjugate results in free saccharide, whereas no saccharide will be released following the same cleavage in a cross-linked conjugate. Likewise, loss of saccharide may result in a detectable decrease in hydrodynamic size of the conjugate, which can be monitored by size exclusion chromatography (SEC-HPLC-RI/UV/MALLS), but this effect may be masked by the concomitant unfolding of the conjugate or aggregation giving rise to an apparent increase in size of the conjugate. Detailed stability studies are performed on the monovalent bulk, which serves several purposes: appropriate methods of physicochemical control are developed, the optimal storage conditions, and shelf life for the bulk is established, and it informs the expected stability of the conjugate in the drug product such that the formulation can be optimized. Accelerated stability studies may provide additional supporting evidence of the stability of the product, but cannot replace real

time studies. The analytical challenges presented by complex of multivalent conjugate vaccines, particularly if adjuvanted, may mean that data on the stability of each monovalent bulk conjugate is used instead.

The stability of the saccharide component of a conjugate vaccine is based on the repeating unit structure, and may be predicted from the wealth of information published on the stability of the parent polysaccharide. Many of the depolymerization mechanisms have been elucidated by the use of NMR spectroscopy, which is a useful tool for monitoring saccharide integrity and the appearance of end groups due to degradation. For Hib, the major depolymerization pathway at neutral or basic pH is by cleavage of the phosphodiester linkage with the participation of a neighboring hydroxyl group, to form a cyclic phosphodiester (Egan et al. 1982). This pathway is catalyzed by cations including the adjuvant aluminum hydroxide, which has implications for the formulation of combination pediatric vaccines (Sturgess et al. 1999). Under acidic conditions, the principal route for depolymerization is through hydrolysis of the glycosidic linkage of the β-Ribf residue, leading to formation of reducing terminal sugar residues and ribitol-5-phosphate end groups (Ravenscroft et al. 2000). This approach is used for the preparation of Hib oligosaccharides suitable for activation and conjugation to CRM197 carrier protein (Costantino et al. 1999).

The major depolymerization pathway for meningococcal group C, Y, and W polysaccharides is through hydrolysis of the labile sialic acid keto linkage, at a rate dependant on pH and temperature. This hydrolysis is controlled to produce oligosaccharides for conjugation (Costantino et al. 1999; Bardotti et al. 2005). The second change in structure may be the loss or migration of *O*-acetyl groups present on these saccharides. These subtle changes can be readily detected by NMR spectroscopy (Jones and Ravenscroft 2008), but it is not yet known if such changes affect the immunogenicity of conjugate vaccines. Group A polysaccharide is the most unstable of the meningococcal polysaccharides, hydrolytic cleavage at the anomeric phosphodiester linkage occurs to generate ManNAc reducing end group and the ManNAc-6-monophosphate. A recent study by Berti et al. (2012) showed that the structurally similar group X polysaccharide, which also contains an anomeric phosphodiester linkage [→4)-α-D-Glc*p*NAc-(1 → P→], is much more stable that the group A polysaccharide [→6)-α-D-Man*p*NAc-(1 → P→]. This was explained by the different orientation of *N*-acetyl group at C-2 with respect to phosphate group at α-C-1. For group A, the axial orientation of the mannosamine *N*-acetyl group could assist in the cleavage of the α phosphate group, whereas this is not possible for group X (equatorial orientation of the glucosamine *N*-acetyl group).

An accelerated stability study performed on groups A, C, Y, and W polysaccharides and monitored by SEC-HPLC-UV showed that only group A polysaccharide showed significant degradation at 37 °C, and this was accompanied by a loss of immunoreactivity (von Hunolstein et al. 2003). Some comparative data on conjugates are available. The analytical methods used to monitor the integrity and stability of bulk and formulated meningococcal group A, C, Y, and W conjugate vaccines have been described (Carinci et al. 2012). The study used SEC-HPLC-PDA/RI/MALLS, CZE, and an HPAEC-PAD method for monitoring the total and

free saccharide in the tetravalent vaccine. There was no detectable change in free saccharide content in the final product vaccine when stored at the recommended temperature for more than a year. In another study, meningococcal group A, C, Y, and W conjugates exhibited similar stabilities at 4 °C, but released different amounts of free saccharide at 55 °C (degradation of A > C > W > Y) (Lei et al. 2000b). Unstable antigens can be prepared as lyophilized presentations, and this is used for all currently licensed group A polysaccharide and conjugate vaccines except for MCV4-DT. Chemical approaches to improve the hydrolytic stability of the group A saccharide component include modification of ring hydroxyl groups (Costantino et al. 2008) and synthesis of stabilized derivatives (Teodorović et al. 2006; Torres-Sanchez et al. 2007; Gao et al. 2013).

Little has been published on the stability of the pneumococcal conjugates; however, much is known about the stability of the polysaccharides. The molecular size and antigenic stability of 23 valent pneumococcal polysaccharide vaccine has been followed by use of SEC-HPLC-RI/RN (Sweeney et al. 2000; MacNair et al. 2005). As discussed in Sect. 8.2.4, stability and forced degradation studies performed on PV23 showed a decrease in molecular size for some polysaccharides (Pn 19A, 19F, and 5), whereas the loss in antigenicity for Pn 1, 9V, and 18C was attributed to losses or shifts of *O*-acetyl groups. The instability of polysaccharides is associated with a phosphodiester linkage and conditions for improving their stability (use of DMSO and sucrose) during reductive amination conjugation have been reported (Prasad 2011). The effect of base hydrolysis on the molecular size of these phosphodiester-containing polysaccharides was studied by use of SEC-HPLC-RI/MALLS (Pujar et al. 2004). The relative order of backbone phosphodiester bond instability due to base hydrolysis was 19A > 10A > 19F > 6B > 17F, 20, whereas degradation of side-chain phosphodiester bonds (11A, 15B, 18C, and 23F) was not detected. The precise impact of base treatment on the structural integrity of the polysaccharides requires additional characterization data, such as composition, NMR analysis, and antigenicity, not reported by these authors. One phosphodiester-containing pneumococcal polysaccharide not present in PV23 is serotype 6A. Detailed kinetic studies showed that the 6A polysaccharide was more susceptible to depolymerization by hydrolysis of the phosphodiester linkage than the cross-reactive polysaccharide 6B (Zon et al. 1982), justifying the latter's inclusion in the polyvalent pneumococcal vaccine.

The relative stability of the pneumococcal vaccines is expected to be based on the intrinsic stability of the saccharide, modulated by the conjugation chemistry and conjugate structure. For example, use of periodate activation resulting in oxidation of backbone residues may make them more vulnerable to subsequent hydrolysis, an approach exploited by Smith degradation used for carbohydrate analysis (Cui 2005; Schulz and Rapp 1991), whereas the introduction of isourea substituents on sugar rings may facilitate elimination reactions. The stability protocols published for Hib and meningococcal group C conjugates by NIBSC (National Institute for Biological Standards and Control) have been applied to two pneumococcal conjugate vaccines (carriers CRM197, PD, and DT/TT) in order to determine the comparative stability of the conjugated serotypes (Gao et al. 2009). The study employed SEC-HPLC for

molecular sizing, free saccharide (by HPAEC-PAD), and conformation was monitored by fluorescence spectroscopy. All the bulk conjugate vaccines were stable when stored the recommended temperature (2–8 °C); however, some differences were observed at higher temperatures. A common feature was the stability of serotype 6B and the instability of 19F (as expected from the polysaccharide stability). At 37 °C and above, the PD conjugates tended to form high molecular weight oligomers or 'aggregates', whereas the CRM197 and TT/DT conjugates showed some tendency to form oligomers/'aggregates' and lose high molecular weight material. The stability of multivalent pneumococcal formulations will in general be dictated by the least stable serotypes, which appear to be Pn 6A and 19A.

In summary, since the drug substance often represents a hold point in the manufacturing process of glycoconjugates, it is important to store it under conditions that ensure its stability so that when the drug product is formulated, the longest possible shelf life is achievable. Instability of the conjugate due to aggregation or adsorption to surfaces play a role, but the most important reason for loss of stability is the hydrolytic degradation of the saccharide, which must be prevented or minimized. Thus, analytical tests to monitor the free saccharide are of crucial importance since this parameter is considered an indicator of vaccine potency and stability, and is a critical factor used to establish the expiry date for the product.

8.6 Formulated and Multivalent Vaccine (Drug Product)

8.6.1 Introduction

The success of vaccines in preventing infectious diseases has resulted in a high number of vaccine administrations during the first 2 years of children's lives. Thus, it is highly desirable to consolidate multiple vaccines into a single formulation and to study the effect and interactions of antigens on the immune response and safety. Typically, combination vaccines are composed of multiple antigens that protect against different diseases (e.g., diphtheria, tetanus, and pertussis), multiple antigens against the same disease, or multiple serotypes/strains of the same disease (or multivalent vaccines, e.g., pneumococcal vaccines). The ambitious target for the vaccine community is to combine ALL childhood vaccines into a single administration but, so far, we have not come anywhere near to achieving that goal.

An important consideration when developing combination vaccines is the effect that one antigen may have on the immune response to another. This applies not only to antigens within a particular vaccine, but also to coadministration of different vaccines. One example is the differences observed in the antibody responses to different capsular-type polysaccharides in the conjugate pneumococcal vaccines in which the magnitude of the titers can show large differences from one serotype to another. The antibody responses can also vary depending on the age of the subject.

Besides having multiple antigens present often at a low concentration the complexity of drug product formulations is augmented by the presence of other components such as adjuvants, stabilizers, excipients, detergents, and/or preservatives. As previously mentioned, polysaccharide–protein conjugates are very complex and tend to push analytical methods to their limits of performance. The problems encountered to test the monovalent drug substance are greatly amplified when these same methods are applied to testing multivalent drug products (DPs). Although any of the DP components may challenge the analytics, it is often the adjuvant in the formulation that has the largest effect. This is true especially if the product interacts with the adjuvant (e.g., aluminum salts) and to a lesser degree if there is little or no binding of the antigen to the adjuvant (e.g., unconjugated CpG oligodeoxynucleotides). Even for relatively simple analysis, sample treatment may be required to avoid interference by other drug product components. This applies to the antigen(s), adjuvant, and excipients for which release or stability testing is required.

Polysaccharide–protein conjugates share all of the concerns associated with polysaccharide vaccines with the added need to monitor the protein component. For polysaccharide vaccines, the active component composition, identity, integrity, and size are considered important quality attributes that can be measured by techniques such as NMR, HPLC, and colorimetric tests. Analytical control strategies for conjugates should include monitoring of chemical modifications of both the polysaccharide and the protein. Quite frequently, a challenge faced is that methods applied to the polysaccharide or protein is not suitable for monitoring these components in the conjugate or to monitor the conjugate as a whole.

It may be tempting to think of the conjugate protein as a "carrier" with a secondary role in the immune response, but the protein selected for conjugation may have an important biological effect. For example for a tetravalent *H. influenzae* protein D pneumococcal conjugate vaccine it was shown that the optimal carrier may be different for each serotype. Comparisons made using conjugates prepared with diphtheria and tetanus carriers showed better responses to serotypes 3, 9V, and 14 in infants when conjugated to diphtheria toxoid (DT) (Nurkka et al. 2001). But a broad case to use DT as carrier could not be made because for serotype 4 polysaccharides exhibited the opposite effect (Ahman et al. 1996). For other serotypes, no difference in immune response was observed.

8.6.2 Manufacturing of Combination Vaccines Drug Product

Since combination vaccines are a collection of individual vaccines mixed together, one of the critical steps in manufacturing is the blending of various antigens, adjuvant, buffer salts, and other excipients to generate a consistent product. For polysaccharide–protein conjugates the appropriate amount to be added is typically determined using the polysaccharide concentration (rather than the protein concentration) as measured by colorimetric or immunochemical methods. Minimizing

assay variability for the chosen concentration method is key to developing a consistent manufacturing process.

Once the various amounts of the vaccine components are determined, they are mixed together under stirring and aseptic conditions to generate a homogeneous product. The bulk drug product requires mixing, filling, handling, and storage that are deeply influenced by the vaccine components, especially the type of adjuvant used. The presence of some adjuvants does not necessarily affect the physical state of the vaccine (e.g., CpGs, saponin QS21), as it remains a true (or near true) solution. Other adjuvants transform the product into a suspension (e.g., aluminum salts). In that case, achieving a homogeneous filling (i.e., a similar amount of vaccine is delivered into each container) becomes more challenging and a balance between keeping the bulk drug product in suspension should be balanced with overmixing that may affect product quality. In addition, the antigen(s) may be partially or (nearly) completely adsorbed to the aluminum salts, creating a dynamic system that may require fine control. Binding to Al salt adjuvants believed to be necessary for immunopotentiation (e.g., depot effect) can prevent antigen loss due to adsorption to container surfaces. Some preclinical evidence, however, appear to contradict the depot effect hypothesis as a mechanism of action for the adjuvant (Noe et al. 2010) showing that antigen binding is not a requirement to generate higher titers in rats (only the presence of the Al containing adjuvant). After filling into the appropriate containers, release testing is performed using suitable methods as described in the next section.

8.6.3 Typical Tests for Multicomponent Formulations

The tests and limits of release and stability require agreement between the sponsor and the regulatory agencies, which can vary from country-to-country or from one region to another. There are general guidelines and, for some vaccines (e.g., conjugated pneumococcal), specific requirements or monographs (WHO and Pharmacopoeia) that should be followed unless a different approach is justified. The tests below represent some of the typical assays used for analytical vaccine testing.

Identification for each polysaccharide present is typically performed by use of a suitable serological or immunochemical method (e.g., slot blot). As for all identity tests, it is necessary to show assay specificity, which can be complicated when the antigens show cross-reactivity. The identification test must be shown to be specific (positive) for each of the active vaccine components and negative for all the others, including other antigens manufactured in the same facility.

The concentration of all drug product components should be properly controlled. This includes the active ingredient(s), adjuvant and excipients. For conjugated polysaccharide–protein vaccines, the saccharide content for each serotype is measured by a suitable immunochemical method such as nephelometry, using reagents (antibodies) that are specific for each antigen. Thus, the total polysaccharide content methods often used for the drug substance cannot be used for the drug product as

the concentration of each individual antigen needs to be shown to be within acceptable ranges. According to the Pharmacopoeia guidelines for pneumococcal conjugated vaccines, the vaccine must contain between 70 and 130 % of the polysaccharide content stated on the label (Anonymous 2013b). This range takes into account both analytical and process variability components. If the method uses antigen specific antibodies to create a standard curve, a strategy to maintain and replace the antigen standard and the antibodies is essential during development and through the lifecycle of the product. The protein concentration is also measured and reported. For aluminum salt-based adjuvants, the concentration is measured by determining the amount of aluminum present (e.g., by titration or ICP-OES). The maximum aluminum content for the adjuvant is 1.25 mg per single human dose (Anonymous 2009) or less (e.g., 0.85 mg/dose), depending on governing agency requirements (Anonymous 2013a).

Two attributes at the top of the criticality assessment list described by the WHO and Pharmacopoeia guidelines are usually conjugate size and antigenicity. For the monovalent drug substance, size can be measured by determining the K_D using size exclusion chromatography and a suitable detection system, such as refractive index, or by using a MALLS detector in tandem with the chromatography system. A colorimetric test can also be used if fractions are collected. For polysaccharide–-protein conjugates, the nephelometry assay can serve as the bioassay or potency assay. These approaches, however, are not suitable for analysis of the combination vaccines because each of the conjugates need to be assessed individually (i.e., changes to the size of each antigen should be measured). Online detection could be problematic due to lack of selectivity of the detector. An approach used for some vaccines includes fraction collection and then analysis of the individual fractions to reconstruct the elution curve for each antigen. This can be done by hydrolyzing the polysaccharide and then analyzing specific sugar components by methods such as HPAEC-PAD or by immunochemical assays (e.g., nephelometry). By standardizing the chromatographic result using K_D, changes during storage can be detected. An added benefit of using an immunochemical assay is that often the same method is used for potency/antigenicity determinations. If that is the case, then size and potency changes can be measured simultaneously. In practice, though, only relatively major changes to these parameters are detectable due to assay limitations such as poor to modest selectivity and relatively high variability. Methods development and data interpretation may be difficult for size determination of combination vaccines formulated in adjuvants such as aluminum salts because the conjugates have to be desorbed first. Recovery and sample integrity after desorption are key parameters that need to be evaluated for these vaccines. Most tests used for the analysis of biological products are not based on “stand alone” methods (i.e., they are not absolute methods and may require support from other techniques or from orthogonal methods). That is the case for size determination of conjugate vaccines. Since the K_D or apparent molecular size depends on the actual size and the shape of the molecule, it can be theorized that a decrease in size (e.g., polysaccharide hydrolysis) could be masked by an increase in unfolding (e.g., stretching of the carrier protein). Two assays that can complement or reveal changes in mass

are an increase of free polysaccharide and/or free protein in the drug product. The main challenge is to develop assays with acceptable degrees of precision, accuracy, quantitation limits, and selectivity (needed to identify which of the conjugates may be degrading).

Safety tests include sterility, bacterial endotoxins, pyrogenicity, general safety, and abnormal toxicity (depending on the region). Pyrogenicity, general safety, and abnormal toxicity are tested during clinical trials with the goal of removing these tests at commercialization if supported by the data. These tests are usually the same for single or multicomponent vaccines. Adjuvant effects must be taken into consideration (especially when the adjuvant generates a biological response).

8.6.4 Formulated Vaccine Critical Quality Attributes and Stability

It has been established that for most of the conjugated vaccines immunogenicity depends on a few critical physicochemical characteristics. In general, the carrier protein, size of the polysaccharide, conjugation chemistry (and degree of conjugation), and the availability of protective epitopes may all impact the immune response to the vaccine.

For most biologics, determining and assigning critical quality attributes to the product is an exercise that spans the drug product, drug substance, intermediates (if present) and key raw materials. The same is true for vaccines, except that for the drug product the number of quality attributes that can be reliably and consistently measured may be very limited. For this reason, critical quality attributes may need to be tested at the drug substance stage only.

Polysaccharide–protein conjugates tend to be fairly complex and heterogeneous molecules, even at the monovalent bulk conjugate stage. Heterogeneity tends to increase as the conjugate degrades, making it difficult to develop suitable stability indicating methods. Since combination vaccine drug products contain multiple antigens at lower concentration than the drug substance, this challenge is exacerbated. Thus, a good potency assay is key in evaluating the stability of the drug product.

A common approach to mitigate the uncertainties of quality attribute criticality and overall stability of drug product is to extrapolate from observations made for the drug substance. Valuable data can be generated by preparing mock drug product containing individual antigens at increased antigen(s) concentration (extrapolating by showing little or no concentration effect on the stability of the molecule) under conditions similar to those intended for DP storage. Establishing the degradation paths for the polysaccharide and protein before conjugation and for the conjugate in simpler environments and under accelerated conditions can point to assays/attributes that should be monitored in the drug product. The effect of the adjuvant should be explored regardless of the approach used, including monitoring for physicochemical, potency, and stability changes. An excellent example of the

adjuvant (aluminum hydroxide) affecting the stability of the antigen/conjugate is provided by Sturgess et al. (1999). The authors uncovered an aluminum catalyzed depolymerization of the polyribosylribitolphosphate (PRP) polysaccharide used to prepare a vaccine against *H. influenza*e type b (Hib).

In summary, combination vaccines drug products present a number of challenges for manufacturing and testing that is in direct proportion to the number of vaccine components, including antigens, adjuvants, and other formulation excipients. Testing is complicated by the need for selective techniques (especially for detection), the (usually) low concentration of the antigens, and the presence of binding adjuvants. These issues and assay variability may seriously limit the use of physicochemical methods to monitor properties such as lot consistency and stability.

8.7 Future Directions

The conjugation technologies, analytics, and regulatory framework established for Hib, meningococcal and pneumococcal vaccines discussed in this chapter are being applied to develop glycoconjugate vaccines against additional strains of these bacteria as well as other pathogens. The vaccine targets are based on the surface carbohydrates of bacteria, fungi, and viruses and require new conjugation strategies combined with improved analytical techniques.

8.7.1 Glycoconjugate Vaccines in Development

8.7.1.1 Extended Coverage of Licensed Polysaccharide and Conjugate Vaccines

Salmonella typhi

Salmonella typhi is a gram-negative bacterium responsible for typhoid fever, a serious disease with high incidence especially in developing countries (Crump et al. 2004). *S. typhi* has a capsular polysaccharide (Vi), which is a linear homopolymer of α-1,4-N-acetylgalactosaminouronic acid with 60–90 % of O-acetylation in position C-3 (Stone and Szu 1988). A polysaccharide-based vaccine is licensed in many countries, but as for other polysaccharide vaccines, it is indicated for active immunization above 2 years of age. Vi polysaccharide–protein conjugates are being developed and draft WHO guidelines have been prepared (Anonymous 2013c); the conjugates are expected to have improved immunogenicity and efficacy in young children and infants. Conjugates of Vi polysaccharide with recombinant exotoxin A of *Pseudomonas aeruginosa* (rEPA) have been shown immunogenic and efficacious in 2–5 year old children (Lin et al. 2001). Currently, Vi-TT and Vi-rEPA conjugates are licensed in India and China, respectively, while a Vi-DT conjugate is

in development (MacLennan et al. 2014). *Citrobacter freundii* produces a polysaccharide antigenically identical to Vi (Snellings et al. 1981), the low health risk of this bacterial strain makes it ideal for use in vaccine manufacturing. More recently, CRM197 has been proposed as a carrier for C. *freundii* derived polysaccharide and a number of papers have been published describing preclinical and characterization studies of CRM197-Vi conjugates. The Vi-CRM vaccine was shown to be safe and highly immunogenic in human clinical studies (Micoli et al. 2011a, 2012; Van Damme et al. 2011) and the technology is being transferred to a manufacturer (MacLennan et al. 2014). Protein capsular matrix vaccine (PCMV) technology in which the polysaccharide is entrapped by a "net" formed by the crosslinking of protein molecules may provide a simpler alternative to well-characterized chemical conjugation. Following promising preclinical data in mice, a Vi construct is being tested in phase 1 clinical trials (Killen et al. 2013).

Neisseria meningitidis

The epidemiology of meningococcal disease features unpredictable epidemics, and variation in endemic serogroup distributions (Halperin et al. 2012), highlighting the importance of developing multivalent vaccine formulations to confer protection against the most relevant serogroups (Harrison et al. 2011). Although five serogroups, Mn A, B, C, W, and Y, cause the majority of meningococcal meningitis (Chang et al. 2012), recently serogroup X has been reported to have the potential for causing seasonal hyperendemicity similar to Mn A and W in Africa (Xie et al. 2013). A vaccine against this emerging serogroup is not available and may become a public health need following the introduction of a monovalent Mn A-TT conjugate vaccine (MenAfriVac®) in the meningitis belt. The serogroup X capsular polysaccharide is a polymer of *N*-acetylglucosamine-4-phosphate residues linked by α-(1 → 4) phosphodiester bonds (Bundle et al. 1973, 1974a, b). The structural similarity to serogroup A suggests the possibility of serogroup replacement following the widespread introduction of MenAfriVac®. Interestingly, the Mn X saccharide is more stable in aqueous solution than Mn A, probably because the equatorial orientation of the *N*-Acetyl group in position 2 does not favor the assistance of the cleavage of the α phosphate group (Berti et al. 2012; Xie et al. 2013). This finding offers the potential for developing a vaccine in a stable liquid formulation. Researchers in the academic and industrial settings are evaluating strategies to develop a conjugate vaccine against serogroup X. Mn X oligosaccharides of varying chain length were conjugated to CRM197 and the conjugates were shown to be immunogenic and to induce bactericidal antibodies in mice (Micoli et al. 2013a). A pentavalent MenACYWX vaccine is also in development, led by the Meningitis Vaccine Project (www.dfid.gov.uk).

Concepts for vaccine prevention of Mn X disease include a monovalent conjugate as well as trivalent AWX or pentavalent ACWYX glycoconjugates combinations (Xie et al. 2013). Preliminary attempts to synthesize Mn X oligosaccharides led to the obtainment of monomer, dimer, and trimer that were conjugated to a protein carrier (Morelli and Lay 2013; Morelli et al. 2014). An attractive new

concept to produce such oligosaccharides is the enzymatic synthesis catalyzed by recombinant meningococcal glycosyl transferases. The capsule polymerases catalyze the sequential addition to the nonreducing ends of short oligosaccharide primers of monosaccharides UDP activated at their reducing ends. If the primers are designed in order to contain a conjugatable linker at their reducing ends, the chemoenzymatically produced saccharides are ready for conjugation to a suitable carrier protein (Fiebig et al. 2014; McCarthy et al. 2013; Mosley et al. 2010; Muindi et al. 2013; Romanow et al. 2013).

Streptococcus pneumoniae

The introduction of pneumococcal conjugate vaccines into childhood immunization schedules has led to a significant decrease in invasive pneumococcal diseases (IPD) in targeted population and unvaccinated adults. However, an increase in disease caused by non-vaccine serotypes such as Pn 22F and 33F has led to their inclusion in a developmental 15-valent pneumococcal conjugate vaccine (PCV15). PCV15 uses periodate oxidation/reductive amination chemistry for conjugation to CRM197 and the vaccine is formulated with $AlPO_4$. PCV15 was shown to be immunogenic in infant rhesus monkeys and is currently in human clinical trials (Ginsburg and Alderson 2011; Skinner et al. 2011). The approval of PCV13 for use in adults together with the variable increases in non-vaccine-serotype disease and possible serotype replacement anticipated in the future (Feikin et al. 2013) is expected to drive the development of higher valency PCVs.

8.7.1.2 New Capsular or Cell Surface Polysaccharides-Based Glycoconjugate Vaccines

Group B Streptococcus

Group B Streptococcus (GBS) is a leading cause of severe bacterial infections in the first 3 months of life and of septic morbidity among mothers, but is also an important cause of morbidity and mortality among other groups and adults with underlying medical conditions (Schuchat 1998; Skoff et al. 2009). Nine GBS serotypes have been recognized based on their expression of distinct capsular polysaccharides: serotypes Ia, Ib, and III are currently the most epidemiologically relevant, although serotypes II and V are also important (Lancaster et al. 2011). The structure of these five serotypes is determined by a different display (or assembly) of the same common sugars Glc, Gal, GlcNAc, and NeuAc (Cieslewicz et al. 2005). The polysaccharides are generally poor immunogens due to their similarity to human tissue antigens (Zou and Jennings 2009).

The conjugation chemistry is generally based on controlled periodate oxidation of terminal sialic acid present in the polymer side chains. The reactive aldehyde groups generated are amenable to reductive amination/conjugation to carrier protein

amino groups. In one example, the ends groups of enzymatically generated type III GBS oligosaccharides have been derivatized and coupled to TT (Paoletti et al. 1990). Conjugates of GBS polysaccharides type Ia, Ib, II, III, and V with TT and CRM197 have been shown to be immunogenic and protective in preclinical testing and human clinical trials have confirmed their safety and immunogenicity (Baker and Edwards 2003; Lancaster et al. 2011; Paoletti and Kasper 2003; Paoletti et al. 1992a, b). Phase III clinical trials of the CRM197 trivalent conjugate are being planned (Madhi et al. 2013). The final goal is a multivalent formulation of the most epidemiologically relevant serotypes. NMR analysis of the nine serotypes has been published (Pinto and Berti 2014); the associated analytical challenges of GBS-CV are similar to those faced by the PCV combinations of conjugate vaccines.

Staphylococcus aureus

Staphylococcus aureus is an encapsulated bacterium, which is a major cause of nosocomial and community-acquired infections; a rapid global increase in drug resistance has added urgency to the need for an effective vaccine (Laxminarayan et al. 2013). Carbohydrate antigens found on the *S. aureus* outer surface include the capsular polysaccharides (CP) of serotype 5 (CP5) or 8 (CP8) and/or a second antigen, a β-(1 → 6)-polymer of *N*-acetyl-D-glucosamine (PNAG). Glycoconjugate vaccines containing CP5 and CP8 covalently linked to the recombinant exotoxin of *P. aeruginosa* (rEPA) have been developed and extensively tested in preclinical and clinical trials, including efficacy ones which unfortunately did not meet the clinical end points (Fattom et al. 2004; Shinefield et al. 2002). The vaccine was subsequently redesigned to include capsular polysaccharide conjugates for types 5 and 8, type 336 (teichoic acid), and two inactivated secreted toxins, α-hemolysin and Panton–Valentine leukocidin. Other approaches include capsular polysaccharides (CP5 and CP8) conjugated to a carrier protein (CRM197) in combination with the recombinant form of the surface exposed *Staphylococcus aureus* proteins clumping factor A (ClfA) and manganese transport protein C (MntC). Clinical studies are currently ongoing (Broughan et al. 2011; Anderson et al. 2012). Recent attempts involve the use of *S. aureus* derived proteins ClfB or IsdB (one of the proteins encoded by the iron-regulated surface determinant system) as the protein carrier for CP5 and CP8 (Pozzi et al. 2012). The use of PNAG as an antigen has also been proposed: the protective epitope appears to reside in the *N*-deacetylated portion of the polymer and synthetic PNAG *N*-deacetylated derivatives conjugated to TT have been reported to induce functional antibodies in mice (Gening et al. 2010). The concept of bio-conjugation, which is described in more detail in Sect. 7.1.3 for *Shigella*, has also been applied to obtain CP5 and CP8 staphylococcal conjugates which induced functional and protective immunity in mice (Wacker et al. 2014).

Group A Streptococcus

Group A *Streptococcus* (GAS) infections are a major cause of morbidity and mortality worldwide with clinical manifestation ranging from pharyngitis and invasive disease to acute rheumatic fever, rheumatic heart disease, and acute post-streptococcal glomerulonephritis (Steer et al. 2009). GAS is a gram-positive microorganism characterized by a capsule of hyaluronic acid (Stollerman and Dale 2008) whose "self" nature hampers its use as a vaccine. GAS bacteria also express the group polysaccharide [→3)-α-L-Rha*p*(1 → 2)[β-D-Glc*p*NAc(1 → 3)]-α-L-Rha*p*(1→]$_n$ which is conserved in the vast majority of strains, and has been considered an attractive target for vaccine development. Human anti-GAS-PS antibodies have been associated with protection against GAS infections (Sabharwal et al. 2006; Salvadori et al. 1995). One concern in using this GAS cell wall carbohydrate as a vaccine derives from the reported cross-reaction with some human tissue (Malkiel et al. 2000; Shikhman et al. 1993). However, the antibodies induced in rabbits by a GAS-PS TT conjugate did not show a significant reaction with several types of human tissue (Sabharwal et al. 2006). An immunodominant doubly branched hexasaccharide epitope has been identified in the structure of the GAS carbohydrate (Michon et al. 2005). Conjugates of both bacteria-derived GAS carbohydrate as well as GAS synthetic oligosaccharides with TT and CRM197 have been prepared and showed to induce protective antibodies in mice and rabbits (Auzanneau et al. 2013; Kabanova et al. 2010b; Sabharwal et al. 2006). The availability of well-defined synthetic structures permitted investigation of the effect of variables such as oligosaccharide length, nature of the non-reducing end and degree of glycosylation degree on the immunogenicity of GAS conjugates (Kabanova et al. 2010b).

Clostridium difficile

Clostridium difficile is a gram-positive microorganism, which is increasingly responsible for healthcare-acquired diarrhea. The incidence of this microorganism is increasing in hospitals worldwide as a consequence of the widespread use of broad-spectrum antibiotics. Two large exotoxins A (TcdA) and B (TcdB) are the major virulence factors of *C. difficile*. A limited number of isolates also produce a binary toxin (CDT), whose patho-physiological role is not clear. *C. difficile* expresses surface polysaccharides, PSI, PSII, and PSIII, which have been isolated and characterized. One of them, PSII, is shared among a number of pathogenic strains including the virulent ribotype 027 which is responsible of outbreaks in US and Canada, in addition it might be part of *C. difficile* biofilm composition (Dapa et al. 2013; Ganeshapillai et al. 2008). To date most of the efforts to develop an anti *C. difficile* vaccine are targeting the toxins and clinical trials of a two component toxoid vaccine are ongoing (Anosova et al. 2013; Foglia et al. 2012; Greenberg et al. 2012; Tian et al. 2012). It seems logical to also consider the surface polysaccharides as possible vaccine candidates, formulated as glycoconjugates to

increase their immunogenicity. PSII is composed of a hexaglycosyl phosphate repeating unit: [→6)-β-D-Glc*p*-(1 → 3)-β-D-Gal*p*NAc-(1 → 4)-α-D-Glc*p*-(1 → 4)-[β-D-Glc*p*-(1 → 3)]-β-D-Gal*p*NAc-(1 → 3)-α-D-Man*p*-(1-P→] (Ganeshapillai et al. 2008). Several CRM197 conjugates have been prepared using: PSII extracted by acidic treatment from *C. difficile* cells, a synthetic hexasaccharide repeating unit with or without phosphorylation in position 6 of the non-reducing glucose as well as a synthetic tetrasaccharide fragment of the repeating unit. Only conjugates of native PSII and the phosphorylated synthetic hexasaccharide conjugate induced antibodies in mice. The antibodies recognized the native polysaccharide and were able to label *C. difficile* bacteria in confocal immunofluorescence microscopy, indicating that the minimal immunogenic structure is the phosphorylated repeating unit (Adamo et al. 2012). In another study, synthetic non-phosphorylated PSII hexasaccharide was conjugated to CRM197 and immunized mice produced anti hapten antibodies; the saccharide structure was also recognized by anti *C. difficile* IgA present in the stools of infected people (Oberli et al. 2011). The repeating unit of PSI [α-L-Rha*p*-(1 → 3)-β-D-Glc*p*-(1 → 4)-[α-L-Rha*p*-(1 → 3)]-α-D-Glc*p*-(1 → 2)-α-D-Glc*p*-(1→], which is expressed by the virulent strain ribotype 027, has been synthesized and coupled to a subunit of *C. difficile* exo-toxin B with the concept of a dual vaccine; the construct was able to detect anti PSI IgG in horse serum (Jiao et al. 2013). PSI synthetic oligosaccharides have been conjugated also to CRM197 and the disaccharide Rha-(1 → 3)-Glc has been identified as the minimal epitope capable of inducing antibodies against the pentasaccharide repeating unit (Martin et al. 2013). Recently PSII polysaccharide has been conjugated to recombinant fragments of the two main *C. difficile* toxins TcdA and TcdB. Both conjugates were able to induce specific anti PSII antibodies and the carrier proteins elicited IgG with neutralizing activity against the corresponding toxins (Romano et al. 2014). Most of the work carried out so far on carbohydrate-based *C. difficile* vaccines has been recently reviewed (Monteiro et al. 2013). Lipoteichoic acid (LTA) has been identified as an alternative surface exposed antigen that is conserved among several strains (Reid et al. 2012). *C. difficile* lipoteichoic acid (LTA) conjugated to a genetically inactivated *P. aeruginosa* exotoxin A resulted in glycoconjugates that induced significant titers of antibodies in mice and rabbits (Cox et al. 2013). The antibodies recognized LTA and *C. difficile* bacteria of a variety of strains and immunofluorescence studies on a single rabbit serum showed binding to both vegetative cells and spores of *C. difficile*. None of the approaches described have been tested in protection experiments; however, the research performed has established the basis for well-defined synthetic conjugate vaccines containing the major forms of *C. difficile* cell surface polysaccharides and will facilitate the development of vaccines if a protective role for these antigens can be demonstrated.

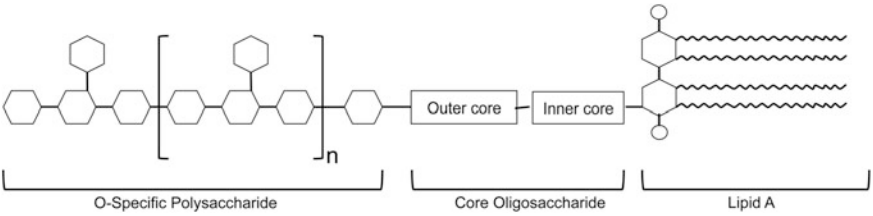


Fig. 8.9 Schematic structure of a lipopolysaccharide showing the O-antigen, core and lipid A regions

8.7.1.3 Lipopolysaccharide-Based Glycoconjugate Vaccines

Lipopolysaccharide (LPS) is one of the major surface components of most gram-negative bacteria and is a virulence factor as well as a protective antigen. LPS consists basically of three regions: the polysaccharide O-antigen, a core carbohydrate containing 10–12 sugar units and a highly toxic and conserved lipid A (Fig. 8.9).

The core is covalently bound through 2-keto-3-deoxyoctonate (KDO) to lipid A. The O-antigen structure contains the antigenic specificity and is the key component of LPS-based vaccines. Due to its toxicity LPS cannot be used for vaccine purposes without prior detoxification to remove the toxic moiety lipid A. This is generally accomplished by acid treatment of LPS which cleaves the linkage between lipid A and KDO. Recently, a new, scalable and possibly general method to purify O-antigens starting from acidic treatment of bacterial cells at the end of fermentation (instead of isolated LPS) has been developed and applied to several *Salmonella* bacteria [*S. paratyphi* A (O:2), *S. typhimurium* (O:4,5), and *S. enteritidis* (O:9)] (Micoli et al. 2013b, 2014). The O-antigens have been conjugated to carrier proteins to enhance their immunogenicity. Many O-antigen-based conjugate vaccines are in development against several human pathogens including *Shigella*, *Salmonellae*, and *E. coli*; some of these studies are reviewed here.

Shigella

Shigella species comprise a family of gram-negative bacteria responsible for serious diarrhea and dysentery. *Shigella* is readily transmitted and represents a serious health issue in developing countries and for travelers (Phalipon et al. 2008; Vinh et al. 2009). One of the approaches to develop a vaccine against *Shigella* is based on the use of the major protective antigen, LPS. The O-polysaccharide, obtained after acid hydrolysis, is conjugated to a carrier protein in order to convert it to a T-cell dependent immunogen. Accordingly, conjugates of the epidemiologically most relevant *Shigella* serotypes (*Shigella dysenteriae* type 1, *Shigella flexneri* type 2a, and *Shigella sonnei*) have been prepared and tested in both preclinical as well as in clinical studies (Chu et al. 1991; Taylor et al. 1993). After preliminary studies to

compare different conjugation strategies in terms of immunogenicity in mice, the chemistry chosen was based on the CNBr mediated derivatization of the O-polysaccharide with ADH, followed by conjugation to the protein carrier (TT or rEPA) using EDAC as the condensing agent. The conjugates with rEPA as carrier have been more extensively studied and were shown to be immunogenic in adults and children; in particular, a *S. sonnei* O-polysaccharide-rEPA conjugate was able to confer protection to adults in a context of shigellosis outbreak (Cohen et al. 1997). Due the complexity of O-polysaccharide purification and conjugation, researchers have also focused on synthetic mimics of the bacterial O-antigen. Phalipon et al. found that a synthetic sequence containing three repeating units of the O-polysaccharide of *S. flexneri* 2a LPS is the best functional mimic of the native polysaccharides and conjugates of this compound conjugated to TT were able to induce protective antibodies in mice (Phalipon et al. 2009). The multistep chemical synthesis leading to the trimer has been recognized as feasible at a large scale with reasonable costs opening the possibility for a future synthetic approach at an industrial scale (Phalipon et al. 2008). In terms of quality control, a well-defined conjugate would facilitate testing of key parameters such degree of glycosylation, size of conjugate, free saccharide, and analysis of glycosylation sites.

Recently, a new approach called bio-conjugation has received increasing attention. The approach is based on glyco-engineering the N-glycosylation pathway in bacteria such as *E. coli* (Fig. 8.1, approach (v)). The polysaccharide, synthesized by enzymes encoded by the inserted genes, is produced on a polyisoprenoid carrier and then an oligosaccharyltransferase is able to transfer the polysaccharide from the carrier lipid to an asparagine residue of the carrier protein which has to contain at least one (native or engineered) N-glycosylation site (Feldman et al. 2005, Ihssen et al. 2010, Kowarik et al. 2006). This technology is being applied to the development of a multivalent *Shigella* conjugate vaccine. A bio-conjugate using rEPA as the carrier was produced against *S. dysenteriae* type 1. It was tested in 40 healthy volunteers and found to be well tolerated and immunogenic (Gambillara 2012). As for the well-defined synthetic oligosaccharide conjugates, the *S. dysenteriae* bio-conjugate was amenable to detailed characterization including size analysis (by HPLC, electrophoretic techniques and MALDI-TOF-MS) and 2D NMR methods to show the structural identity/integrity of the saccharide moiety (Ravenscroft et al. 2012). Similarly a bio-conjugate against *S. flexneri* type 2a has been developed and characterized and is being prepared for phase I clinical trials (Kowarik et al. 2012).

Salmonella paratyphi A and Nontyphoidal *Salmonella*

The major cause of enteric fever in certain areas of Asia and India is *S. paratyphi* A, while infections from *S. typhimurium* and *S. enteritidis* are more common in developed countries and Africa, where they are an important cause of bacteremia in children. These types of *Salmonella* do not have a capsular polysaccharide, and the O specific polysaccharide (O-SP) present in the LPS of these gram-negative bacteria, following appropriate detoxification by acidic treatment, is being used to develop

conjugate vaccines. The *S. paratyphi* A O-SP has a the following repeating unit → 2)-[α-D-Par*p*-(1 → 3)]-α-D-Man*p*-(1 → 4)-α-L-Rha*p*3OAc-(1 → 3)-[α-D-Glcp-(1 → 6)]-α-D-Gal*p*-(1 → where paratose (3,6-dideoxy-D-ribohexopyranose) confers the antigenic specificity; the O-acetylation in position 3 of Rha has been reported to be critical for immunogenicity (Konadu et al. 1996). John Robbins's team compared different models of *S. paratyphi* A O-SP conjugates in mice, two based on specific terminal KDO modification and one based on random activation of the polysaccharide chain with CNBr, in both cases the activated saccharides were coupled to TT. Based on the preclinical results, only a conjugate model based on random activation chemistry mediated by CDAP (a more practical and safer reagent than CNBr) was selected for further clinical studies, which demonstrated its safety and immunogenicity/protection (Konadu et al. 1996, 2000). Recently a different model of *S. paratyphi* A O-SP conjugate based on selective modification of KDO and conjugation to the carrier protein CRM197 to obtain a highly immunogenic construct has been proposed (Micoli et al. 2011a, b). *S. typhimurium* and *S. enteritidis* O-antigens share a common backbone with *S. paratyphi* A, but differ in the structure of the branches, in particular the substitutions at position 3 of mannose, abequose, tyvelose or paratose, respectively, confer serological specificity (Hellerqvist et al. 1968; Konadu et al. 1996; Micoli et al. 2013b; Rahman et al. 1997). A vaccine program for typhoidal and non typhoidal *Salmonella enterica* serovars including conjugates of *S. typhimurium* and *S. enteritidis* O-SP is being developed (Martin 2013; Podda 2010; MacLennan et al. 2014). The concept of obtaining more effective protection through use of a *Salmonella*-derived protein as carrier is also being investigated (Simon et al. 2011, 2013). Strategies for the development of glycoconjugate vaccines against *Salmonella* have been reviewed by Simon and Levine (2012).

Vibrio cholerae

Cholera is still a major cause of morbidity and mortality in the developing world. Infection is acquired from contaminated water or food and can be rapidly spread following war or natural disasters as demonstrated by the cholera outbreak in Haiti that followed the earthquake in 2010. The majority of *Vibrio cholerae* clinical infections are caused by serogroups O1 and O139. Serogroup O1 has two serotypes: Inaba and Ogawa. A parenteral whole cell *V. cholerae* vaccine was used during the first half of the twentieth century; however, it was characterized by a short duration of protection and adverse reactions and was removed from licensing in the 1970s (Ftacek et al. 2013; Girard et al. 2006). Despite the presence of killed or live attenuated vaccines, a subunit cholera vaccine would be desirable because of its expected higher manufacturing consistency and level of characterization. The LPS of O-SP of Ogawa and Inaba O1 serotypes consists of (1 → 2)-α-linked 4-amino-4,6-dideoxy-D-mannose (perosamine), whose amino group is acylated with 3-deoxy-L-glycero-tetronic acid (Hisatsune et al. 1993; Ito et al. 1994; Kenne et al. 1982). O139 strains produce an LPS molecule that lacks the O-antigen composed of

a perosamine homopolymer and is antigenically distinct from O1 (Cox and Perry 1996). *V. cholerae* LPS has been shown to be involved in protective immunity elicitation in humans and experimental animals (Jonson et al. 1996; Losonsky et al. 1997; Qadri et al. 1999; Svennerholm and Holmgren 1976). The teams of John Robbins and Fournier showed that detoxified O1 LPS- Cholera toxin and O139 LPS-TT conjugates elicited vibriocidal antibodies in mice and humans (Boutonnier et al. 2001; Gupta et al. 1992, 1998). An approach that would circumvent the need for LPS detoxification and result in more defined and reproducible conjugates is based on the use of synthetic LPS fragments conjugated to protein carriers. Paul Kovac's team generated a considerable amount of data on conjugates composed of the terminal hexasaccharide fragments of Ogawa and Inaba LPS conjugated to BSA or rEPA. The general outcome of these studies is that Ogawa synthetic conjugates elicit significant titers of vibriocidal (protective) antibodies in animal models, whereas Inaba conjugates did not induce protective immunity (Chernyak et al. 2002; Meeks et al. 2004; Saksena et al. 2005, 2006; Wade et al. 2006). More recently, Kovac's team used squarate coupling chemistry to prepare different molecular models of conjugates with the recombinant tetanus toxin HC fragment as carrier. These included synthetic monovalent constructs (Ogawa or Inaba) or bivalent conjugates where synthetic Ogawa and Inaba hexasaccharides were linked to the same carrier molecule; in addition, they also prepared a conjugate bearing the synthetic Ogawa hexasaccharide and a tetrapeptide glycan as molecular adjuvant on the same carrier molecule (Bongat et al. 2010). The dependence of saccharide chain length and density of glycosylation on the immunogenicity of Ogawa synthetic O1-TT conjugates has been studied using hexa-, octa- and decasaccharide synthetic fragments conjugated via a 17-atom linker (Ftacek et al. 2013). A length dependence of immune response was observed with the longer decasaccharide conjugates inducing the highest anti-LPS IgG level in mice. The immune response also appeared to be dependent on the saccharide loading density: in general, it increased with increasing loading peaking at an optimal loading of a molar ratio of 230 ± 10 monosaccharide units per TT, after which a decline in the immune response was observed. Despite a certain level of cross-reactive Inaba LPS antibodies, the corresponding vibriocidal activity was very low or absent.

The synthetic approach for O-specific polysaccharide fragment of O139 *V. cholerae* is more challenging since its repeating unit is a complex branched hexasaccharide consisting of five different monosaccharides, two rare deoxy sugars and a 4,6 cyclic phosphate and more work seems to be required to produce oligosaccharides that can be conjugated and tested in animal model (Ruttens and Kováč 2006; Turek et al. 2006). Although the synthetic approach has the potential to become a viable strategy to develop well-defined and protective conjugates against *V. cholerae*, further work is still needed to improve the protective ability of Inaba conjugates and to obtain O139 synthetic conjugates.

Bordetella pertussis

Current vaccination against *B. pertussis* is based on acellular pertussis (aP) or cellular pertussis (wP) vaccines combined with diphtheria and tetanus toxoids to give DTaP or DTwP, which might include also other antigens conferring protection against other pathogens, such as poliovirus, hepatitis B, and Hib. The aP based vaccines contain different subunit antigens depending on the manufacturer, but the main component is pertussis toxin (PT), which may be genetically, or chemically detoxified. However, the recent resurgence of *B. pertussis* infections in US and Europe (Celentano et al. 2005; Cherry 2012) indicates the need to improve vaccination schedules as well as current vaccines. One approach involving the glycoconjugate concept consists of coupling a dodecasaccharide obtained by acid hydrolysis of *B. pertussis* or *B. bronchiseptica* LPS, to an aminooxy derivative of the protein carrier to give a stable oxime involving the reducing end of the oligosaccharide. The oligosaccharides and resulting conjugates have been well characterized, and when tested in mice, were able to induce specific anti LPS antibodies and bactericidal titers (Kubler-Kielb et al. 2011).

8.7.1.4 Glycoconjugates Against Fungal Pathogens

Carbohydrate structures dominate the surface of fungal pathogen and a number of investigations are ongoing to identify protective carbohydrate epitopes on pathogenic fungi such as *Candida* and *Cryptococcus neoformans.*

Candida

Candida species are among the most common causes of bloodstream infections in nosocomial settings. There is increasing evidence that the cell surface β-1,2 mannans and β-1,3 glucans polymers of *C. albicans* and most pathogenic fungi, respectively, contain protective epitopes (Bromuro et al. 2002; Nitz et al. 2002). CRM197 conjugates of β-1,3 glucans containing sporadic β-1,6 branches derived from the alga *Laminaria digitata* (Laminarin), linear β-1,3 glucans from *Alcaligenes faecalis* (Curdlan), as well as branched and linear synthetic β-1,3 glucans have been shown to be immunogenic and protective in mice. Interestingly the synthetic linear structures showed better protection profiles compared to the branched ones indicating that the protective epitope might be linear (Bromuro et al. 2010; Torosantucci et al. 2005). Synthetic dimers and trimers of β-1,2 mannans have been shown to be good inhibitors of *C. albicans* protective monoclonal antibodies. The mannans have been used to prepare conjugates to carrier proteins as well as a synthetic peptide; the conjugates were shown to be immunogenic and protective in mice and rabbits (Bundle et al. 2012; Lipinski et al. 2012; Trkola et al. 1996; Wu and Bundle 2005; Wu et al. 2007; Xin et al. 2008). Bundle's team improved the immunogenicity of the synthetic glycopeptide β-1,2 mannan trisaccharide-Fba, by

coupling this construct to TT. The resulting conjugate was able to induce protective immunity in mice without the aid of dendritic cell-based immunization, required when the glycopeptide was administered alone (Xin et al. 2012). In another approach to further improve the immunogenicity of the β-1,2 mannan trisaccharide-TT conjugate, Bundle's team designed a tricomponent anti *C. albicans* vaccine. The researchers covalently linked both the β-1,2 mannan trisaccharide and the β-1,3 glucan laminarin to the same TT molecule which, besides containing a protective anti-candida epitope as discussed above, is also known to be a ligand of the pattern recognition receptor Dectin-1 and could therefore activate innate immunity pathways (Lipinski et al. 2013).

Cryptococcus neoformans

Cryptococcus neoformans is a fungal pathogen, which primarily affects immunocompromised subjects, but can also be a cofactor in pulmonary disease for normal individuals (Arora and Huffnagle 2005; Casadevall and Pirofski 2005). Cryptococcal meningitis is a leading cause of death among persons living with HIV/AIDS in sub-Saharan Africa. *C. neoformans* has a polysaccharide capsule that is an important virulence factor; it is composed of mostly glucuronoxylomannan (GXM) and to a lesser extent by galactoxylomannan (GalXM) and mannoproteins (MP). The structure of GXM is very complex and consists of a linear α-1,3 mannan trisaccharide with side groups of β-1,2 Glc*p*A and β-1,2, and β-1,4 Xyl*p*, attached at different mannoses of the repeating units to give up to six different structures, which are called chemotypes (described as serotypes A–C and AD) (Cherniak et al. 1998). Additional structural complexity and heterogeneity is introduced by O-acetylation. GalXM represents about 7 % of the capsular mass and is made of a backbone of α-1,6 galactan with potentially four short oligosaccharide branch structures. Early attempts to make an anti *Cryptococcus* polysaccharide–protein conjugate vaccine resulted in a poorly characterized product, which showed immunogenicity in mice (Goren and Middlebrook 1967). Subsequently GXM-TT conjugates, more suitable to further preclinical and clinical development, were shown to be immunogenic and protective in mice (Devi 1996; Devi et al. 1991). GXM is a complex antigen and can induce both protective and non-protective antibodies (Mukherjee et al. 1995). In order to identify the structure of the protective epitope, which resides in serotype A GXM, a number of structures were synthesized and tested for their ability to bind with monoclonal antibodies (Oscarson et al. 2005). More recently, a synthetic M2 serotype A heptasaccharide identified from this study was conjugated to HSA; unfortunately although the conjugate elicited antibodies in mice, they were not protective (Nakouzi et al. 2009). Similarly GalXM conjugates induced antibodies in mice, but they were not protective (Chow and Casadevall 2011). Further studies seem necessary to elucidate the nature of the protective epitopes present in the capsule of *C. neoformans* and synthetic oligosaccharides might help in answering this question.

8.7.1.5 Conjugate Vaccines Against Viral Infectious Diseases

Viruses are characterized by surface carbohydrates linked to viral proteins by means of the host glycosylation machinery. Consequently the oligosaccharides, which decorate viral proteins, may appear as "self" to the immune system, thus providing a strong defense against host immune surveillance. Thus, the use of viral oligosaccharides for vaccine development may present a risk because of the potential to induce auto immunity reactions. However, there are situations where particular "nonself" epitopes are created on the viral surface and the recapitulation of such epitopes by synthetic or biological procedures may constitute an attractive vaccine strategy. One example of a carbohydrate-based protective viral epitope can be found in the human immunodeficiency virus HIV-1 envelope. A broad neutralizing antibody (bNAb), 2G12, isolated from an infected individual (Trkola et al. 1996) specifically binds an oligomannose cluster on the HIV-1 envelope glycoprotein gp120, and has a specific and unusual Fab structure characterized by a V_H domain exchange that creates an extended antibody binding site surface (Calarese et al. 2003). A number of attempts to reproduce the structural and antigenic characteristics of oligomannose-based 2G12 epitope have been reported using different strategies. Once demonstrated that the synthetic constructs efficiently bound 2G12 directly or by inhibition of the 2G12/gp120 binding, candidate vaccines have been prepared by linking the artificial 2G12 epitopes to carrier proteins and their immunogenicity has been tested in animal models. While in most cases antibodies that recognized the carbohydrate hapten were induced, in all cases these antibodies failed to bind gp120 in vitro and/or did not show activity in neutralizing tests demonstrating the difficulty of inducing Fab swapped-domain antibodies (Astronomo et al. 2008, 2010; Doores et al. 2010; Kabanova et al. 2010a; Ni et al. 2006). Recently, other and more potent bNAbs, which are strictly glycan-dependent, have been isolated and appear to target conserved glycopeptide epitopes (McLellan et al. 2011; Pejchal et al. 2011). Further research on the structural features of the epitopes recognized by these new bNAb might allow the preparation of a protective anti-HIV vaccine containing these critical carbohydrate epitopes. Current strategies to develop carbohydrate-based anti-HIV vaccines have been comprehensively reviewed by Wang et al. (2014).

8.7.1.6 New Approaches to Conjugation and New Carriers in Development

The concept of bio-conjugation already described for *Shigella* conjugates is also being applied to the development of vaccines against other pathogens including *Staphylococcus aureus* (Lee et al. 2011; Wacker et al. 2014), *P. aeruginosa*, *Salmonella*, *E. coli* and *S. pneumoniae*. A recent approach aimed to provide flexibility and versatility in the preparation of conjugate vaccines formulations is represented by Multiple Antigen-Presenting System (MAPS), which is based on an affinity coupling approach. The target carrier/antigen proteins, genetically fused to a biotin-binding

protein, spontaneously attach to a biotinylated polysaccharide via a strong affinity linkage, resulting in a macromolecular construct, which can potentially display multiple protein antigens along the polysaccharide backbone (Zhang et al. 2013).

Protein carriers currently used in licensed glycoconjugate vaccines are TT, DT, CRM197, OMPC, and the recently introduced protein D. Other proteins are increasingly used as carrier and some of them have also been tested in clinical trials. A notable example is rEPA, which has been used as carrier for *Shigella* O-antigens and *Salmonella* Vi conjugates, is also used as a carrier for bio-conjugates. A recombinant protein (N19) containing promiscuous human CD4+ T-cell epitopes derived from various pathogens, proved to be a very good carrier for Hib oligosaccharides and meningococcal oligosaccharides suggesting the potential of N19 to be a candidate carrier protein for future or optimized conjugate vaccines (Baraldo et al. 2004; Falugi et al. 2001). An emerging concept is the utilization of carrier proteins, which can also contribute to protection against the target pathogen or another pathogen (Ginsburg and Alderson 2011; Pozzi et al. 2012; Romano et al. 2014). In this case, the carrier protein has to provide not only T cell helper epitopes, but also protective B cell epitopes, therefore the design of the conjugation and the control of the product are of paramount importance in order to avoid loss of protective epitopes on the protein. Approaches to obtain well-defined conjugates have been recently reviewed (Berti and Adamo 2013). Epitope mapping of carrier proteins using structural biology tools like the crystal structure of protein/antibody complexes or Hydrogen Deuterium Exchange coupled to Mass Spectrometry could inform the chemist on how to develop specific, critical epitope preserving and scalable glycosylation methods (Malito et al. 2013). Biophysical and immunochemical methods need to be in place to confirm the presence of intact protein and saccharide B epitopes on the conjugate. Finally, the concept of hapten-carrier conjugate is now being evaluated to develop vaccines against noninfectious diseases, like cancer, alzheimer, drug abuse, etc. (Costantino et al. 2011) and other carrier proteins such as keyhole limpet hemocyanin (KLH) and the virus-like particle Qb are being used to developing such conjugate vaccines (Danishefsky and Allen 2000; Winblad et al. 2012). Recombinant protein-based viral vaccines are discussed in Chap. 3.

8.7.2 Analytical Techniques in Development

A plethora of techniques for the biophysical and immunochemical characterization of glycoproteins (glycomics and proteomics) are being improved or developed; some advances in the field and potentially applicable to glycoconjugates vaccines are highlighted here.

8.7.2.1 Saccharide Structure by NMR Spectroscopy

NMR spectroscopy plays an important role in antigen identification and research into interaction with proteins. Elucidation of the primary structure of saccharide antigens by use of 1D and 2D NMR experiments is well established and facilitated by the employment of NMR chemical shift predictions programs (Lundborg and Widmalm 2011). Additional NMR experiments to obtain $^{1}H,^{1}H$ cross-relaxation rates, coupling constants and $^{1}H,^{13}C$- and $^{1}H,^{1}H$-residual dipolar couplings combined with molecular dynamics simulations can be used to identify possible antigen conformations (Widmalm 2013). The conformation of the antigen when bound to protein can be determined by transferred NOE NMR experiments and the binding specificity explored using saturation transfer difference NMR experiments. These techniques can be employed to better understand antigen-antibody interactions and optimize vaccine development.

Improvements in NMR hardware (field strength, nano- and cryoprobes) and software (pulse-sequence design and spectral processing) permit increasingly complex mixtures of small amounts of material to be characterized and quantified. The applications of NMR to biopharmaceuticals, from drug discovery to quality control of formulated products, has been recently reviewed by Wishart (2013). Although the identity/structure of the saccharide component can be profiled throughout the conjugation process by use of 1D ^{1}H NMR spectroscopy, spectral overlap of saccharide signals is a problem even at high field. This can be circumvented by exploitation of the spectral resolution possible in the ^{13}C dimension allowing the saccharide component of the glycoconjugate to be fully characterized by 2D ^{1}H-^{13}C HSQC maps (Ravenscroft et al. 2012). The use of optimized heteronuclear NMR spectroscopy for the fast and accurate quantitation of glucans in complex mixtures together with the use of reference libraries has been described (Bøjstrup et al. 2013; Petersen et al. 2013) and could be applied to carbohydrate vaccines and possibly multivalent combinations.

In addition to the liquid-state NMR spectroscopy applications discussed, the introduction of high-resolution magic-angle sample spinning (HR-MAS) probes has extended use of this technology to semi-solids. This means that high resolution NMR can also be performed on bacterial cells and purification intermediates, not possible using liquid state NMR. Thus, the detailed structure of the surface carbohydrates on the cell can be characterized and then tracked through to the purified polysaccharide. By identifying the "true structure" of the surface antigen, this approach may also help to identify immunological epitopes important for vaccine development, in particular when chemical modifications, such as *O*-acetyl loss or migration may occur during the purification process.

HR-MAS NMR has been used for on-cell analysis of surface carbohydrates, such as polysaccharides, O-antigens, and lipotechoic acids. HR-MAS analysis of meningococcal group A bacteria determined the precise structure of the capsular polysaccharides expressed on the cell surface (Gudlavalleti et al. 2006). Using a capsule-defective strain, the study showed that O-acetylation is not necessary for capsular polysaccharides surface transport and also that the O-acetylation on living

cells agree well with that of purified polysaccharides. HR MAS has been used to track the evolution of O-antigen structure, particularly the pattern of O-acetylation, on live *S. typhimurium* bacteria (Zandomeneghi et al. 2012). A subsequent study proved that the appearance of de-*O*-acetylated O-antigen in the stationary growth phase was due to the de-O-acetylation of already synthesized *O*-acetylated O-antigen caused by the metabolism-induced basic pH of the growth medium (Ilg et al. 2013). These experiments show the structural power of HR MAS and demonstrate that it can be used to screen bacterial strains and identify possible vaccine targets as has been reported for *Clostridium difficile* (Adamo et al. 2012; Reid et al. 2012).

When the pathogenic nature of the bacteria involved in the production of polysaccharides makes a direct analysis of bacterial slurry problematic, development of similar methods at the earliest stage of the purification or fermentation process, with minimal manipulations or pretreatments, could be necessary. Moreover, this method can provide important information such as whether to proceed with the purification of the capsular polysaccharide in the corresponding fermentation batch and also support validation and optimization the fermentation process. For anionic polysaccharides such as PRP present in *H. influenzae* type b, where the first isolation step is typically the fractional precipitation by formation of water-insoluble salts or complexes, HR-MAS can be developed as an identity assay of the capsular polysaccharide present in such precipitates without extensive manipulations or pretreatments Wieruszeski et al. 2005).

8.7.2.2 Improved Characterization of Solution Properties of Carbohydrate-Based Vaccines

As for polysaccharide vaccines, important physical parameters that are routinely monitored for conjugate preparations are molecular size and molecular size distribution performed by use of size exclusion chromatography with multiple detectors SEC-HPLC-UV/RI/MALLS. A recent innovation has been the use of a reverse phase (RP) column for size-based separation of hydrophilic polysaccharides; the study included an investigation of stationary and mobile phase parameters and the coupling of RP-HPLC to MALLS (He et al. 2014). Several techniques that give additional structural information and do not require the limitation of elution through columns have been described for polysaccharides (Harding et al. 2010, 2012) and proteins (Abdelhameed et al. 2012; Chaudhuri et al. 2013; Hu et al. 2013) and are potentially applicable to glycoconjugates. These orthogonal methods and their application are discussed in detail for polysaccharides (Chap. 7). They include analytical ultracentrifugation, viscometry, and light scattering, and can be used to characterize the hydrodynamic properties of conjugates. Such techniques are particularly important for the analysis of very large cross-linked conjugates (and aggregates) and to assist in formulation and stability studies.

8.7.2.3 Improved Chromatographic Techniques

Most of the technologies potentially applicable to glycoconjugates vaccines are being developed for the rapidly burgeoning fields of glycomics and proteomics. The glycosylation analysis of glycoproteins involves three different approaches: (i) characterization of glycans in intact glycoproteins, (ii) characterization of glycopeptides and (iii) structural analysis of chemically or enzymatically released glycans. HILIC and capillary electrophoresis (of fluorescently-labeled released glycans) are widely used to determine the detailed sequence and linkage analysis of released glycans (Mittermayr et al. 2013). The so-called N-glycan "nanoprofiling" has been reported, which uses nanoLC-coupled electrospray ionization (ESI)–MS with an intercalated nanofluorescence reader, and provides effective single glycan separation with subpicomolar sensitivity (Kalay et al. 2012).

HPAEC-PAD of unlabeled glycans is routinely performed as HPAEC permits unique separation of carbohydrates including isomers at high pH, while PAD provides sensitive detection of picomole amounts of underivatized glycans. One of the major limitations of this technique is that identification of unknown eluting carbohydrates is not easily derived from retention times. For such identification, fractions containing the carbohydrates have to be desalted (using a carbohydrate membrane desalter or anion-self-regenerating suppressor) followed by fraction collection and NMR or MS analysis. Such procedures are time-consuming and not suitable for routine analysis of carbohydrates. As for RP-HPLC and HILIC, which are now routinely coupled to MS, development of robust hyphenation of HPAEC-PAD with mass spectrometry is in progress (Rohrer et al. 2013). The development of a capillary HPAEC-PAD-MS system delivers both the desired sensitivity, as well as online direct structural analysis of oligosaccharides (or monosaccharides) by mass spectrometry. Analysis of urinary oligosaccharides by a prototype system of capillary-scale HPAEC-PAD and online electrospray-ionization ion-trap mass spectrometry revealed several new oligosaccharides (Bruggink et al. 2012) and clearly illustrates the potential of such technology for improved characterization of polysaccharides and glycoconjugates vaccines.

8.7.2.4 Multiplex Quantification

Multiplex assays are a new technology using the principles of simultaneous detection and quantification of analytes. Different multiplex systems have become commercially available and are used in a wide variety of protein expression profiling applications, including diagnosis of infectious diseases, cancer, autoimmune and allergic diseases. For example, one of the available multiplex systems is Luminex technology, which is based on fluorescent beads ("microspheres") that are color-coded into 100 distinct sets. Each bead can be coated with a reagent specific for a particular assay, allowing the capture and detection of a specific analyte from the sample. Within the Luminex analyzer, lasers excite the internal dyes that identify each microsphere particle, and also any reporter dye captured during the assay. The

advantages of multiplex assays, compared to conventional tests like ELISA are their greater sensitivity and greater dynamic ranges and it allows the detection of multiple analytes in a single sample simultaneously. Moreover, multiplex assays are also cost- and time-effective, and minimize the sample volume requirements. Multiplex assays are already used for measurement of serotype-specific pneumococcal capsular polysaccharide IgGs (Goldblatt et al. 2011; Klein et al. 2012) and their development for identity and quantification tests of polysaccharides present in multivalent conjugate vaccines will facilitate the quality control of these vaccines.

8.8 Conclusions

Glycoconjugate vaccines against *H. influenzae* type b, *N. meningitidis*, *S. pneumoniae* have been licensed and strategies for their manufacturing, testing, and regulation established. The success of these vaccines opens up exciting possibilities for the control of many infectious diseases caused by encapsulated pathogens. The conjugation platform of methodologies and techniques established have facilitated the development of conjugate vaccines against additional strains of these bacteria and other pathogens including *Streptococcus, Staphylococcus* and enteric bacteria as well as fungi such as *C. albicans and C. neoformans*. These challenges are being met by the development of new conjugation strategies combined with improved analytical techniques and new approaches to making well-defined conjugates using synthetic and biosynthetic methods.

Acknowledgments One of us (NR) would like to thank PATH and all the vaccine manufacturers who have made available samples of polysaccharides and glycoconjugate vaccines and for the many fruitful discussions, as well as the assistance of students Kirstin Anderson, Taigh Anderson, and Carey Pike with checking the manuscript and references.

References

Abdelhameed AS, Morris GA, Adams GG, Rowe AJ, Laloux O, Cerny L, Bonnier B, Duvivier P, Conrath K, Lenfant C, Harding SE (2012) An asymmetric and slightly dimerized structure for the tetanus toxoid protein used in glycoconjugate vaccines. Carbohydr Polym 90 (4):1831–1835

Abeygunawardana C, Williams TC, Sumner JS, Hennessey JP Jr (2000) Development and validation of an NMR-based identity assay for bacterial polysaccharides. Anal Biochem 279:226–240

Adam O, Moreau M (2000) Improved methods for the quantification of free and linked spacer in conjugate vaccine process. Dev Biol (Basel) 103:105–111

Adamo R, Romano MR, Berti F, Leuzzi R, Tontini M, Danieli E, Cappelletti E, Caciki OS, Swennen E, Pinto V, Brogioni B, Proietti D, Galeotti CL, Lay L, Monteiro MA, Scarselli M, Costantino P (2012) Phosphorylation of the synthetic hexasaccharide repeating unit is essential for the induction of antibodies to *Clostridium difficile* PSII cell wall polysaccharide. ACS Chem Biol 7:1420–1428. doi:10.1021/cb300221f

Ahman H, Käyhty H, Tamminen P, Vuorela A, Malinoski F, Eskola J (1996) Pentavalent pneumococcal oligosaccharide conjugate vaccine PncCRM is well-tolerated and able to induce an antibody response in infants. Pediatr Infect Dis J 15:134–139

Alexander HE, Heidelberger M, Leidy G (1944) The protective or curative element in type b *H. influenzae* rabbit antiserum. Yale J Biol Med 16:425–434

Alexander HE, Leidy G (1947) The present status of treatment for influenzal meningitis. Am J Med 2(5):457–466

Anderson PW, Pichichero ME, Insel RA, Betts R, Eby R, Smith DH (1986) Vaccines consisting of periodate-cleaved oligosaccharides from the capsule of *Haemophilus influenzae* type b coupled to a protein carrier: structural and temporal requirements for priming in the human infant. J Immunol 137(4):1181–1186

Anderson AS, Miller AA, Donald RG, Scully IL, Nanra JS, Cooper D, Jansen KU (2012) Development of a multicomponent *Staphylococcus aureus* vaccine designed to counter multiple bacterial virulence factors. Hum Vaccin Immunother 8(11):1585–1594

Anonymous (2000) Recommendations for the production and control of *Haemophilus influenzae* type b conjugate vaccines. WHO Tech Rep Series 897:27–56

Anonymous (2004) Recommendations for the production and control of meningococcal group C conjugate vaccines. WHO Tech Rep Series 924:102–128

Anonymous (2005) Recommendations for the production and control of pneumococcal conjugate vaccines. WHO Tech Rep Series 927:64–98

Anonymous (2006) Recommendations to assure the quality, safety and efficacy of group A meningococcal conjugate vaccines. WHO Tech Rep Series 962:117–171

Anonymous (2008) European pharmacopoeia monograph for *Haemophilus* type b conjugate vaccine. 01/2008:1219

Anonymous (2009) Recommendations to assure the quality, safety and efficacy of pneumococcal conjugate vaccines. World Health Organization. http://www.who.int/biologicals/areas/vaccines/pneumo/Pneumo_final_23APRIL_2010.pdf. Accessed 13 June 2013

Anonymous (2013a) General chapter <1234> vaccines for human use—polysaccharide and glycoconjugate vaccines, U.S. Pharmacopeia and the National Formulary (USP–NF)

Anonymous (2013b) European pharmacopoeia monograph for pneumococcal polysaccharide conjugate vaccines (adsorbed) 01/2013:2150

Anonymous (2013c) Recommendations to assure the quality, safety and efficacy of typhoid conjugate vaccines. World Health Organization. http://www.who.int/biologicals/areas/vaccines/TYPHOID_BS2215_doc_v1.14_WEB_VERSION.pdf Accessed 14 July 2014

Anosova NG, Brown AM, Li L, Liu N, Cole LE, Zhang J, Mehta H, Kleanthous H (2013) Systemic antibody responses induced by a two-component *Clostridium difficile* toxoid vaccine protect against *C. difficile*-associated disease in hamsters. J Med Microbiol 62:1394–1404

Arora S, Huffnagle GB (2005) Immune regulation during allergic bronchopulmonary mycosis: lessons taught by two fungi. Immunol Res 33(1):53–68. doi:10.1385/IR:33:1:053

Ashwell G (1957) Colorimetric analysis of sugars. Method Enzymol 3:73–105

Astronomo RD, Kaltgrad E, Udit AK, Wang SK, Doores KJ, Huang CY, Pantophlet R, Paulson JC, Wong CH, Finn MG, Burton DR (2010) Defining criteria for oligomannose immunogens for HIV using icosahedral virus capsid scaffolds. Chem Biol 17(4):357–370. doi:10.1016/j.chembiol.2010.03.012

Astronomo RD, Lee H-K, Scanlan CN, Pantophlet R, Huang CY, Wilson IA, Blixt O, Dwek RA, Wong CH, Burton DR (2008) A glycoconjugate antigen based on the recognition motif of a broadly neutralizing human immunodeficiency virus antibody, 2G12, is immunogenic but elicits antibodies unable to bind to the self glycans of gp120. J Virol 82(13):6359–6368. doi:10.1128/JVI.00293-08

Aubin Y, Jones C, Freedberg DI (2010) Using NMR spectroscopy to obtain the higher order structure of biopharmaceutical products. BioPharm Int (Supplement):28–38

Auzanneau FI, Borrelli S, Pinto BM (2013) Synthesis and immunological activity of an oligosaccharide-conjugate as a vaccine candidate against Group A *Streptococcus*. Bioorg Med Chem Lett 23(22):6038–6042

Baker CJ, Edwards MS (2003) Group B streptococcal conjugate vaccines. Arch Dis Child 88 (5):375–378. doi:10.1136/adc.88.5.375

Baraldo K, Mori E, Bartoloni A, Petracca R, Giannozzi A, Norelli F, Rappuoli R, Grandi G, Del Giudice G (2004) N19 polyepitope as a carrier for enhanced immunogenicity and protective efficacy of meningococcal conjugate vaccines. Infect Immun 72:4884–4887. doi:10.1128/IAI.72.8.4884-4887.2004

Bardotti A, Averani G, Berti F, Berti S, Carinci V, D'Ascenzi S, Fabbri B, Giannini A, Maganoli C, Proietti D, Norelli F, Rappuoli R, Ricci S, Costantino P (2008) Physicochemical characterization of glycoconjugate vaccines for prevention of meningococcal diseases. Vaccine 26(18):2284–2296

Bardotti A, Averani G, Berti F, Berti S, Galli C, Giannini S, Fabbri B, Proietti D, Ravenscroft N, Ricci S (2005) Size determination of bacterial capsular oligosaccharides used to prepare conjugate vaccines against *Neisseria meningitidis* groups Y and W135. Vaccine 23 (16):1887–1899

Bardotti A, Proietti D, Ricci S (2012) Analysis of saccharide vaccines without interference. US Patent 8,137,680, 20 March 2012

Bardotti A, Ravenscroft N, D'Ascenzi S, Averani G (2000) Quantitative determination of saccharide in *Haemophilus influenzae* type b glycoconjugate vaccines by high-performance anion-exchange chromatography with pulsed amperometric detection. Vaccine 18:1982–1993

Bastos RC, De Carvalho JM, Da Silveira IAFB, do Couto Jacob S, Leandro KC (2010) Determination of hydrazine in a meningococcal C conjugate vaccine intermediary product. Vaccine 28(34):5648–5651

Bednar B, Hennessey JP Jr (1993) Molecular size analysis of capsular polysaccharide preparations from *Streptococcus pneumoniae*. Carbohydr Res 243(1):115–130

Belfast M, Lu R, Capen R, Zhong J, Nguyen MA, Gimenez J, Sitrin R, Mancinelli R (2006) A practical approach to optimization and validation of a HPLC assay for analysis of polyribosyl-ribitol phosphate in complex combination vaccines. J Chromatogr B 832:208–215

Berti F, Adamo R (2013) Recent mechanistic insights on glycoconjugate vaccines and future perspectives. ACS Chem Biol 8(8):1653–1663. doi:10.1021/cb400423g

Berti F, Romano MR, Micoli F, Pinto V, Cappelletti E, Gavini M, Proietti D, Pluschke G, MacLennan CA, Costantino P (2012) Relative stability of meningococcal serogroup A and X polysaccharides. Vaccine 30:6409–6415. doi:10.1016/j.vaccine.2012.08.021

Beuvery EC, Miedema F, van Delft R, Haverkamp J (1983a) Preparation and immunochemical characterization of meningococcal group C polysaccharide-tetanus toxoid conjugates as a new generation of vaccines. Infect Immun 40(1):39–45

Beuvery EC, Speijers GJ, Lutz BI, Freudenthal D, Kanhai V, Haagmans B, Derks HJ (1985) Analytical, toxicological and immunological consequences of the use of N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide as coupling reagent for the preparation of meningococcal group C polysaccharide-tetanus toxoid conjugate as vaccine for human use. Dev Biol Stand 63:117–128

Beuvery EC, vd Kaaden A, Kanhai V, Leussink AB (1983b) Physicochemical and immunological characterization of meningococcal group A polysaccharide-tetanus toxoid conjugates prepared by two methods. Vaccine 1:31–36

Black SB, Shinefield HR, Fireman B, Hiatt R, Polen M, Vittinghoff E (1991) Efficacy in infancy of oligosaccharide conjugate *Haemophilus influenzae* type b (HbOC) vaccine in a United States population of 61,080 children. Pediatr Infect Dis J 10:97–104

Bøjstrup M, Petersen BO, Beeren SR, Hindsgaul O, Meier S (2013) Fast and accurate quantitation of glucans in complex mixtures by optimized heteronuclear NMR spectroscopy. Anal Chem 85:8802–8808

Bolgiano B, Mawas F, Yost SE, Crane DT, Lemercinier X, Corbel MJ (2001) Effect of physico-chemical modification on the immunogenicity of Haemophilus influenzae type b oligosaccharide–CRM_{197} conjugate vaccines. Vaccine 19(23):3189–3200

Bolgiano B, Mawas F, Burkin K, Crane DT, Saydam M, Rigsby P, Corbel MJ (2007) A retrospective study on the quality of *Haemophilus influenzae* type b vaccines used in the UK between 1996 and 2004. Hum Vaccines 3(5):176–182

Bongat AFG, Saksena R, Adamo R, Fujimoto Y, Shiokawa Z, Peterson DC, Fukase K, Vann WF, Kovac P (2010) Multimeric bivalent immunogens from recombinant tetanus toxin HC fragment, synthetic hexasaccharides, and a glycopeptide adjuvant. Glycoconj J 27:69–77. doi:10.1007/s10719-009-9259-4

Boutonnier A, Villeneuve S, Nato F, Dassyl B, Fournier JM (2001) Preparation, immunogenicity, and protective efficacy, in a murine model, of a conjugate vaccine composed of the polysaccharide moiety of the lipopolysaccharide of *Vibrio cholerae* O139 bound to tetanus toxoid. Infect Immun 69:3488–3493. doi:10.1128/IAI.69.5.3488-3493

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72(1):248–254

Brady C, Killeen K, Taylor W, Patkar A, Lees A (2012) Carrier protein outsourcing. BioProcess Int 10(10):50–55

Bröker M, Costantino P, DeTora L, McIntosh ED, Rappuoli R (2011) Biochemical and biological characteristics of cross-reacting material 197 (CRM_{197}), a non-toxic mutant of diphtheria toxin: use as a conjugation protein in vaccines and other potential clinical applications. Biologicals 39 (4):195–204

Bromuro C, Romano M, Chiani P, Berti F, Tontini M, Proietti D, Mori E, Torosantucci A, Costantino P, Rappuoli R, Cassone A (2010) Beta-glucan-CRM_{197} conjugates as candidates antifungal vaccines. Vaccine 28:2615–2623. doi:10.1016/j.vaccine.2010.01.012

Bromuro C, Torosantucci A, Chiani P, Conti S, Polonelli L, Cassone A (2002) Interplay between protective and inhibitory antibodies dictates the outcome of experimentally disseminated Candidiasis in recipients of a *Candida albicans* vaccine. Infect Immun 70:5462–5470

Broughan J, Anderson R, Anderson AS (2011) Strategies for and advances in the development of *Staphylococcus aureus* prophylactic vaccines. Expert Rev Vaccines 10:695–708. doi:10.1586/erv.11.54

Brown F, Corbel M, Griffiths E (2000) Physico-chemical procedures for the characterization of vaccines. Karger, Basel, p 103 (Dev Biol (Basel))

Bruggink C, Poorthuis BJ, Deelder AM, Wuhrer M (2012) Analysis of urinary oligosaccharides in lysosomal storage disorders by capillary high-performance anion-exchange chromatography–-mass spectrometry. Anal Bioanal Chem 403(6):1671–1683

Bundle DR (2011) New frontiers in the chemistry of glycoconjugate vaccines. In: Bagnoli F, Rappuoli R (eds) Vaccine design: innovative approaches and novel strategies. Academic Press, Norfolk

Bundle DR, Jennings HJ, Kenny CP (1973) An improved procedure for the isolation of meningococcal, polysaccharide antigens, and the structural determination of the antigen from serogroup X. Carbohydr Res 26:268–270

Bundle DR, Jennings HJ, Kenny CP (1974a) Studies on the group-specific polysaccharide of *Neisseria meningitidis* serogroup X and an improved procedure for its isolation. J Biol Chem 249:4797–4801

Bundle DR, Smith IC, Jennings HJ (1974b) Determination of the structure and conformation of bacterial polysaccharides by carbon 13 nuclear magnetic resonance. Studies on the group-specific antigens of *Neisseria meningitidis* serogroups A and X. J Biol Chem 249:2275–2281

Bundle DR, Nycholat C, Costello C, Rennie R, Lipinski T (2012) Design of a *Candida albicans* disaccharide conjugate vaccine by reverse engineering a protective monoclonal antibody. ACS Chem Biol 7(10):1754–1763. doi:10.1021/cb300345e

Cai X, Lei QP, Lamb DH, Shannon A, Jacoby J, Kruk J, Kensinger RD, Ryall R, Zablackis E, Cash P (2004) LC/MS characterization of meningococcal depolymerized polysaccharide group C reducing endgroup and internal repeating unit. Anal Chem 76(24):7387–7390

Calarese DA, Scanlan CN, Zwick MB, Deechongkit S, Mimura Y, Kunert R, Zhu P, Wormald MR, Stanfield RL, Roux KH, Kelly JW, Rudd PM, Dwek RA, Katinger H, Burton DR, Wilson IA (2003) Antibody domain exchange is an immunological solution to carbohydrate cluster recognition. Science 300(5628):2065–2071. doi:10.1126/science.1083182

Calix JJ, Nahm MH, Zartler ER (2011) Elucidation of structural and antigenic properties of pneumococcal serotype 11A, 11B, 11C, and 11F polysaccharide capsules. J Bacteriol 193 (19):5271–5278

Calix JJ, Porambo RJ, Brady AM, Larson TR, Yother J, Abeygunwardana C, Nahm MH (2012) Biochemical, genetic, and serological characterization of two capsule subtypes among *Streptococcus pneumoniae* serotype 20 strains: discovery of a new pneumococcal serotype. J Biol Chem 287(33):27885–27894. doi:10.1074/jbc.M112.380451

Canaán-Haden L, Cremata J, Chang J, Valdes Y, Cardoso F, Bencomo V (2006) High-performance reverse phase chromatography with fluorescence detection assay for characterization and quantification of pneumococcal polysaccharides. Vaccine 24:S70–S71

Capiau C, Deschamps M, Desmons PM, Laferriere CAJ, Poolman J, Prieels JP (2008) Vaccines against antigens from bacteriae. European Patent No. EP 1163000. European Patent Office, Munich, Germany

Carinci V, Bernardini A, Campa C, Magagnoli C, Beccai F, D'Ascenzi S (2012) Stability studies and characterization of meningococcal A, C, Y, and W135 glycoconjugate vaccine. In: Bhattacharyya L, Rohrer JS (eds) Applications of ion chromatography in the analysis of pharmaceutical and biological products. Wiley, Hoboken

Casadevall A, Pirofski L-A (2005) Feasibility and prospects for a vaccine to prevent cryptococcosis. Med Mycol 43:667–680

Celentano LP, Massari M, Paramatti D, Salmaso S, Tozzi AE, EUVAC-NET Group (2005) Resurgence of pertussis in Europe. Pediatr Infect Dis J 24:761–765. doi:10.1097/01.inf.0000177282.53500.77

Chang Q, Tzeng Y-L, Stephens DS (2012) Meningococcal disease: changes in epidemiology and prevention. Clin Epidemiol 4:237–245. doi:10.2147/CLEP.S28410

Chaudhuri R, Cheng Y, Middaugh CR, Volkin DB (2013) High-throughput biophysical analysis of protein therapeutics to examine interrelationships between aggregate formation and conformational stability. AAPS J 16(1):48–64. doi:10.1208/s12248-013-9539-6

Chen PS Jr, Toribara TT, Warner H (1956) Microdetermination of phosphorus. Anal Chem 28 (11):1756–1758

Cherniak R, Valafar H, Morris LC, Valafar F (1998) *Cryptococcus neoformans* chemotyping by quantitative analysis of ^{1}H nuclear magnetic resonance spectra of glucuronoxylomannans with a computer-simulated artificial neural network. Clin Diagn Lab Immunol 5:146–159

Chernyak A, Kondo S, Wade TK, Meeks MD, Alzari PM, Fournier JM, Taylor RK, Kovac P, Wade WF (2002) Induction of protective immunity by synthetic *Vibrio cholerae* hexasaccharide derived from *V. cholerae* O1 Ogawa lipopolysaccharide bound to a protein carrier. J Infect Dis 185:950–962. doi:10.1086/339583

Cherry JD (2012) Epidemic pertussis in 2012—the resurgence of a vaccine-preventable disease. N Engl J Med 367(9):785–787. doi:10.1056/NEJMp1209051

Chow SK, Casadevall A (2011) Evaluation of *Cryptococcus neoformans* galactoxylomannan–-protein conjugate as vaccine candidate against murine cryptococcosis. Vaccine 29 (10):1891–1898. doi:10.1016/j.vaccine.2010.12.134

Chu CY, Liu BK, Watson D, Szu SS, Bryla D, Shiloach J, Schneerson R, Robbins JB (1991) Preparation, characterization, and immunogenicity of conjugates composed of the O-specific polysaccharide of *Shigella dysenteriae* type 1 (Shiga's bacillus) bound to tetanus toxoid. Infect Immun 59:4450–4458

Cieslewicz MJ, Chaffin D, Glusman G, Kasper D, Madan A, Rodrigues S, Fahey J, Wessels MR, Rubens CE (2005) Structural and genetic diversity of Group B *Streptococcus* capsular polysaccharides. Infect Immun 73(5):3096–3103. doi:10.1128/IAI.73.5.3096-3103.2005

Cohen D, Ashkenazi S, Green MS, Gdalevich M, Robin G, Slepon R, Yavzori M, Orr N, Block C, Ashkenazi I, Shemer J, Taylor DN, Hale TL, Sadoff JC, Pavliakova D, Schneerson R, Robbins JB (1997) Double-blind vaccine-controlled randomised efficacy trial of an investigational *Shigella sonnei* conjugate vaccine in young adults. Lancet 349:155–159. doi:10.1016/S0140-6736(96)06255-1

Cook MC, Bliu A, Kunkel JP (2013) Quantitation of serogroups in multivalent polysaccharide-based meningococcal vaccines: optimisation of hydrolysis conditions and chromatographic methods. Vaccine 31(36):3702–3711

Costantino P, Viti S, Podda A, Velmonte MA, Nencioni L, Rappuoli R (1992) Development and phase 1 clinical testing of a conjugate vaccine against meningococcus A and C. Vaccine 10 (10):691–698

Costantino P, Norelli F, Giannozzi A, D'Ascenzi S, Bartoloni A, Viti S, Paffetti R, Bigio M, Pennatini C, Guarnieri G, Gallo E, Averani G, Seid R, Ravenscroft N, Lazzeroni C, Kaur S, Rappuoli R, Ceccarini C (1999) Size fractionation of bacterial capsular polysaccharides for their use in conjugate vaccines. Vaccine 17(9):1251–1263

Costantino P, Rappuoli R, Berti F (2011) The design of semi-synthetic and synthetic glycoconjugate vaccines. Expert Opin Drug Discov 6:1045–1066. doi:10.1517/17460441.2011.609554

Costantino P, Berti F, Norelli F, Bartoloni A (2008) Modified saccharides having improved stability in water. European Patent EP 1490409, 31 Dec 2008

Cox AD, Perry MB (1996) Structural analysis of the O-antigen-core region of the lipopolysaccharide from *Vibrio cholerae* O139. Carbohydr Res 290:59–65

Cox AD, St Michael F, Aubry A, Cairns CM, Strong PC, Hayes AC, Logan SM (2013) Investigating the candidacy of a lipoteichoic acid-based glycoconjugate as a vaccine to combat *Clostridium difficile* infection. Glycoconj J 30:843–855. doi:10.1007/s10719-013-9489-3

Crane DT, Bolgiano B, Jones C (1997) Comparison of the diphtheria mutant toxin, CRM_{197}, with a *Haemophilus influenzae* type-b polysaccharide-CRM_{197} conjugate by optical spectroscopy. Eur J Biochem 246:320–327

Crump JA, Luby SP, Mintz ED (2004) The global burden of typhoid fever. Bull World Health Organ 82:346–353

Cui SW (2005) Structural analysis of polysaccharides. In: Cui SW (ed) Food carbohydrates: chemistry, physical properties, and applications. Taylor & Francis Group, Florida

D'Ambra AJ, Baugher JE, Concannon PE, Pon PA, Michon F (1997) Direct and indirect methods for molar-mass analysis of fragments of the capsular polysaccharides of *Haemophilus influenzae* type b. Anal Biochem 250(2):228–236

Danishefsky SJ, Allen JR (2000) From the laboratory to the clinic: a retrospective on fully synthetic carbohydrate-based anticancer vaccines. Angew Chem Int Ed Engl 39(5):836–863

Dapa T, Leuzzi R, Ng YK, Baban ST, Adamo R, Kuenhne SA, Scarselli M, Minton NP, Serruto D, Unnikrishnan M (2013) Multiple factors modulate biofilm formation by the anaerobic pathogen *Clostridium difficile*. J Bacteriol 195(3):545–555. doi:10.1128/JB.01980-12

de Santana-Filho AP, Noleto GR, Gorin PAJ, de Souza LM, Lacomini M, Sassaki GL (2012) GC–MS detection and quantification of lipopolysaccharides in polysaccharides through 3-O-acetyl fatty acid methyl esters. Carbohydr Polym 87(4):2730–2734

Devi SJ (1996) Preclinical efficacy of a glucuronoxylomannan-tetanus toxoid conjugate vaccine of *Cryptococcus neoformans* in a murine model. Vaccine 14:841–844

Devi SJ, Schneerson R, Egan W, Ulrich TJ, Bryla D, Robbins JB, Bennett JE (1991) *Cryptococcus neoformans* serotype A glucuronoxylomannan-protein conjugate vaccines: synthesis, characterization, and immunogenicity. Infect Immun 59:3700–3707

Dische Z (1947) A new specific color reaction of hexuronic acids. J Biol Chem 167:189–198

Dische Z, Shettles LB (1948) A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination. J Biol Chem 175(2):595–603

Donnelly JJ, Deck RR, Liu MA (1990) Immunogenicity of a *Haemophilus influenzae* polysaccharide-*Neisseria meningitidis* outer membrane protein complex conjugate vaccine. J Immunol 145(9):3071–3079

Doores KJ, Fulton Z, Hong V, Patel MK, Scanlan CN, Wormald MR, Finn MG, Burton DR, Wilson IA, Davis BG (2010) A nonself sugar mimic of the HIV glycan shield shows enhanced antigenicity. Proc Natl Acad Sci USA 107(40):17107–17112. doi:10.1073/pnas.1002717107

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356

Egan W (2000) Physico-chemical characterization of polysaccharide vaccines. Dev Biol (Basel) 103:3–9

Egan W, Frasch CE, Anthony BF (1995) Lot-release criteria, postlicensure quality control and the *Haemophilus influenzae* Type b conjugate vaccines. JAMA 273(11):888–889

Egan W, Schneerson R, Werner KE, Zon G (1982) Structural studies and chemistry of bacterial capsular polysaccharides. Investigations of phosphodiester-linked capsular polysaccharides isolated from *Haemophilus influenzae* types a, b, c, and f: NMR spectroscopic identification and chemical modification of endgroups and the nature of base-catalyzed hydrolytic depolymerization. J Am Chem Soc 104:2898–2910

Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82(1):70–77

Elson LA, Morgan WTJ (1933). A colorimetric method for the determination of glucosamine and chondrosamine. Biochem J 27(6):1824–1828

Falugi F, Petracca R, Mariani M, Luzzi E, Mancianti S, Carinci V, Melli ML, Finco O, Wack A, Di Tommaso A, De Magistris MT, Costantino P, Del Giudice G, Abringnani S, Rappuoli R, Grandi G (2001) Rationally designed strings of promiscuous CD4(+) T cell epitopes provide help to *Haemophilus influenzae* type b oligosaccharide: a model for new conjugate vaccines. Eur J Immunol 31:3816–3824. doi:10.1002/1521-4141(200112)31:12<3816::AID-IMMU3816>3.0.CO;2-K

Farkaš P, Bystrický S (2010) Chemical conjugation of biomacromolecules: a mini-review. Chem Pap 64(6):683–695

Fattom A, Li X, Cho YH, Burns A, Hawwari A, Shepherd SE, Coughlin R, Winston S, Naso R (1995) Effect of conjugation methodology, carrier protein, and adjuvants on the immune response to *Staphylococcus aureus* capsular polysaccharides. Vaccine 13:1288–1293

Fattom AI, Horwith G, Fuller S, Propst M, Naso R (2004) Development of StaphVAX, a polysaccharide conjugate vaccine against *S. aureus* infection: from the lab bench to phase III clinical trials. Vaccine 22:880–887. doi:10.1016/j.vaccine.2003.11.034

Feikin DR, Kagucia EW, Loo JD, Link-Gelles R, Puhan MA et al (2013) Serotype-specific changes in invasive pneumococcal disease after pneumococcal conjugate vaccine introduction: a pooled analysis of multiple surveillance sites. PLoS Med 10(9):e1001517. doi:10.1371/journal.pmed.1001517

Feldman MF, Wacker M, Hernandez M, Hitchen PG, Marolda CL, Kowarik M, Morris HR, Dell A, Valvano MA, Aebi M (2005) Engineering N-linked protein glycosylation with diverse O-antigen lipopolysaccharide structures in *Escherichia coli*. Proc Natl Acad Sci USA 102:3016–3021. doi:10.1073/pnas.0500044102

Fiebig T, Berti F, Freiberger F, Pinto V, Claus H, Romano MR, Proietti D, Brogioni B, Stummeyer K, Berger M, Vogel U, Costantino P, Gerardy-Schahn R (2014) Functional expression of the capsule polymerase of *Neisseria meningitidis* serogroup X: a new perspective for vaccine development. Glycobiology 24(2):150–158. doi:10.1093/glycob/cwt102

Foglia G, Shah S, Luxemburger C, Pietrobon PJF (2012) *Clostridium difficile*: development of a novel candidate vaccine. Vaccine 30(29):4307–4309

Forsgren A, Riesbeck K (2008) Protein D of *Haemophilus influenzae*: a protective nontypeable *H. influenzae* antigen and a carrier for pneumococcal conjugate vaccines. Clin Infect Dis 46 (5):726–731

Fothergill LD, Wright J (1933) Influenzal meningitis: the relation of age incidence to the bactericidal power of blood against the causal organism. J Immunol 24:273–284

Frech C, Hilbert AK, Hartmann G, Mayer K, Sauer T, Bolgiano B (2000) Physicochemical analysis of purified diphtheria toxoids: is toxoided then purified the same as purified then toxoided? Dev Biol (Basel) 103:205–215

Ftacek P, Nelson V, Szu SC (2013) Immunochemical characterization of synthetic hexa-, octa- and decasaccharide conjugate vaccines for *Vibrio cholerae* O:1 serotype Ogawa with emphasis on antigenic density and chain length. Glycoconj J 30:871–880. doi:10.1007/s10719-013-9491-9

Gambillara V (2012) The conception and production of conjugate vaccines using recombinant DNA technology. BioPharm Int 25(1):28–32

Ganeshapillai J, Vinogradov E, Rousseau J, Weese JS, Monterio MA (2008) *Clostridium difficile* cell-surface polysaccharides composed of pentaglycosyl and hexaglycosyl phosphate repeating units. Carbohydr Res 343:703–710. doi:10.1016/j.carres.2008.01.002

Gao F, Lockyer K, Burkin K, Crane DT, Bolgiano B (2009) Pneumococcal conjugate vaccine stability from a physico-chemical point-of-view. Poster presented at meningitis research foundation's 2009 conference—Meningitis and septicaemia in children and adults, royal society of medicine, London, 11–12 Nov 2009

Gao Q, Tontini M, Brogioni G, Nilo A, Filippini S, Harfouche C, Polito L, Romano MR, Costantino P, Berti F, Adamo R, Lay L (2013) Immunoactivity of protein conjugates of carba analogues from *Neisseria meningitidis* A capsular polysaccharide. ACS Chem Biol 8 (11):2561–2567. doi:10.1021/cb400463u

Gening ML, Maira-Litran T, Kropec A, Skurnik D, Grout M, Tsvetkov YE, Nifantiev NE, Pier GB (2010) Synthetic β-(1 → 6)-linked *N*-acetylated and nonacetylated oligoglucosamines used to produce conjugate vaccines for bacterial pathogens. Infect Immun 78:764–772. doi:10.1128/IAI.01093-09

Giannini G, Rappuoli R, Ratti G (1984) The amino-acid sequence of two non-toxic mutants of diphtheria toxin: CRM_{45} and CRM_{197}. Nucleic Acids Res 12(10):4063–4069

Ginsburg AS, Alderson MR (2011) New conjugate vaccines for the prevention of pneumococcal disease in developing countries. Drugs Today 47:207–214. doi:10.1358/dot.2011.47.3.1556471

Girard MP, Steele D, Chaignat CL, Kieny MP (2006) A review of vaccine research and development: human enteric infections. Vaccine 24(15):2732–2750

Goren MB, Middlebrook GM (1967) Protein conjugates of polysaccharide from *Cryptococcus neoformans*. J Immunol 98:901–913

Goebel WF, Avery OT (1931) Chemo-immunological studies on conjugated carbohydrate proteins. V. The immunological specificity of an antigen prepared by combining the capsular polysaccharide of Type III *Pneumococcus* with foreign proteins. J Exp Med 54:437–447

Goldblatt D, Ashton L, Zhang Y, Antonello J, Marchese RD (2011) Comparison of a new multiplex binding assay versus the enzyme-linked immunosorbent assay for measurement of serotype-specific pneumococcal capsular polysaccharide IgG. Clin Vaccine Immunol 18 (10):1744–1751

Gorringe AR, Pajon R (2012) Bexsero: a multicomponent vaccine for prevention of meningococcal disease. Hum Vaccin Immunother 8(2):174–183

Greenberg RN, Marbury TC, Foglia G, Warny M (2012) Phase I dose finding studies of an adjuvanted *Clostridium difficile* toxoid vaccine. Vaccine 30(13):2245–2249

Gudlavalleti SK, Szymanski CM, Jarrell HC, Stephens DS (2006) *In vivo* determination of *Neisseria meningitidis* serogroup A capsular polysaccharide by whole cell high-resolution magic angle spinning NMR spectroscopy. Carbohydr Res 341(4):557–562

Guo YY, Anderson R, McIver J, Gupta RK, Siber GR (1998) A simple and rapid method for measuring unconjugated capsular polysaccharide (PRP) of *Haemophilus influenzae* type b in PRP-tetanus toxoid conjugate vaccine. Biologicals 26(1):33–38

Gupta RK, Szu SC, Finkelstein RA, Robbins JB (1992) Synthesis, characterization, and some immunological properties of conjugates composed of the detoxified lipopolysaccharide of *Vibrio cholerae* O1 serotype Inaba bound to cholera toxin. Infect Immun 60:3201–3208

Gupta RK, Taylor DN, Bryla DA, Robbins JB, Szu SC (1998) Phase 1 evaluation of *Vibrio cholerae* O1, serotype Inaba, polysaccharide-cholera toxin conjugates in adult volunteers. Infect Immun 66:3095–3099

Haan A, Put RM, Beurret M (2013) HPAEC-PAD method for the analysis of alkaline hydrolyzates of *Haemophilus influenzae* type b capsular polysaccharide. Biomed Chromatogr 27 (9):1137–1142

Halperin SA, Bettinger JA, Greenwood B, Harrison LH, Jelfs J, Ladhani SN, McIntyre P, Ramsay ME, Safadi MA (2012) The changing and dynamic epidemiology of meningococcal disease. Vaccine 30(Suppl 2):B26–B36. doi:10.1016/j.vaccine.2011.12.032

Hara S, Takemori Y, Yamaguchi M, Nakamura M, Ohkura Y (1987) Fluorometric high-performance liquid chromatography of *N*-acetyl-and *N*-glycolylneuraminic acids and its application to their microdetermination in human and animal sera, glycoproteins, and glycolipids. Anal Biochem 164:138–145

Harding SE, Abdelhameed AS, Morris GA (2010) Molecular weight distribution evaluation of polysaccharides and glycoconjugates using analytical ultracentrifugation. Macromol Biosci 10 (7):714–720

Harding SE, Abdelhameed AS, Morris GA, Adams G, Laloux O, Cerny L, Bonnier B, Duvivier P, Conrath K, Lenfant C (2012) Solution properties of capsular polysaccharides from *Streptococcus pneumoniae*. Carbohydr Polym 90(1):237–242

Harrison LH, Pelton SI, Wilder-Smith A, Holst J, Safadi MA, Vazquez JA, Taha MK, LaForce FM, von Gottberg A, Borrow R, Plotkin SA (2011) The global meningococcal initiative: recommendations for reducing the global burden of meningococcal disease. Vaccine 29:3363–3371. doi:10.1016/j.vaccine.2011.02.058

Hasija M, Li L, Rahman N, Ausar SF (2013) Forced degradation studies: an essential tool for the formulation development of vaccines. Vaccine Dev Therapy 3:11–13

He Y, Hou W, Thompson M, Holovics H, Hobson T, Jones MT (2014) Size exclusion chromatography of polysaccharides with reverse phase liquid chromatography. J Chromatogr A 1323:97–103. doi:10.1016/j.chroma.2013.11.010

Hellerqvist CG, Lindberg B, Svensson S, Holme T, Lindberg AA (1968) Structural studies on the O-specific side-chains of the cell-wall lipopolysaccharide from *Salmonella typhimurium* 395 ms. Carbohydr Res 8:43–55. doi:10.1016/S0008-6215(00)81689-4

Hepler R, Yu Ip CC (1994) Application of capillary ion electrophoresis and ion chromatography for the determination of o-acetate groups in bacterial polysaccharides. J Chromatogr A 680:201–208

Hestrin S (1949) The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine, and its analytical application. J Biol Chem 180(1):249–261

Hisatsune K, Kondo S, Isshiki Y, Iguchi T, Haishima Y (1993) Occurrence of 2-O-methyl-*N*-(3-deoxy-L-glycero-tetronyl)-D-perosamine (4-amino-4,6-dideoxy-D-manno-pyranose) in lipopolysaccharide from Ogawa but not from Inaba O forms of O1 *Vibrio cholerae*. Biochem Biophys Res Commun 190:302–307. doi:10.1006/bbrc.1993.1046

Ho MM, Bolgiano B, Corbel MJ (2000) Assessment of the stability and immunogenicity of meningococcal oligosaccharide C-CRM_{197} conjugate vaccines. Vaccine 19:716–725

Ho MM, Lemercinier X, Bolgiano B, Crane D, Corbel MJ (2001) Solution stability studies of the subunit components of meningococcal C oligosaccharide–CRM_{197} conjugate vaccines. Biotechnol Appl Biochem 33:91–98

Ho MM, Mawas F, Bolgiano B, Lemercinier X, Crane DT, Huskisson R, Corbel MJ (2002) Physico-chemical and immunological examination of the thermal stability of tetanus toxoid conjugate vaccines. Vaccine 20:3509–3522

Holliday MR, Jones C (1999) Meeting report: WHO-Co-sponsored informal workshop on the use of physicochemical methods for the characterization of *Haemophilus influenzae* type b conjugate vaccines. Biologicals 27:51–53

Hsieh CL (2000) Characterization of saccharide-CRM_{197} conjugate vaccines. Dev Biol (Basel) 103:93–104

Hu L, Joshi SB, Liyanage MR, Pansalawatta M, Alderson MR, Tate A, Robertson G, Maisonneuve J, Volkin DB, Middaugh CR (2013) Physical characterization and formulation development of a recombinant pneumolysoid protein-based pneumococcal vaccine. J Pharm Sci 102(2):387–400

Ihssen J, Kowarik M, Dilettoso S, Tanner C, Wacker M, Thony-Meyer L (2010) Production of glycoprotein vaccines in *Escherichia coli*. Microb Cell Fact 9:61–73. doi:10.1186/1475-2859-9-61

Ilg K, Zandomeneghi G, Rugarabamu G, Meier BH, Aebi M (2013) HR-MAS NMR reveals a pH-dependent LPS alteration by de-O-acetylation at abequose in the O-antigen of *Salmonella enterica Serovar typhimurium*. Carbohydr Res 382:58–64

Ito T, Higuchi T, Hirobe M, Hiramatsu K, Yokota T (1994) Identification of a novel sugar, 4-amino-4,6-dideoxy-2-O-methylmannose in the lipopolysaccharide of *Vibrio cholerae* O1 serotype Ogawa. Carbohydr Res 256(1):113–128

Jiao Y, Ma Z, Hodgins D, Pequegnat B, Bertolo L, Arroyo L, Monteiro MA (2013) *Clostridium difficile* PSI polysaccharide: synthesis of pentasaccharide repeating block, conjugation to exotoxin B subunit, and detection of natural anti-PSI IgG antibodies in horse serum. Carbohydr Res 378:15–25. doi:10.1016/j.carres.2013.03.018

Johnson I, Spence MTZ (2010) The molecular probes handbook. A guide to fluorescent probes and labeling technologies, 11th edn. Molecular Probes, Eugene

Jones C (2005) NMR assays for carbohydrate-based vaccines. J Pharm Biomed Anal 38 (5):840–850

Jones C, Lemercinier X (2002) Use and validation of NMR assays for the identity and O-acetyl content of capsular polysaccharides from *Neisseria meningitidis* used in vaccine manufacture. J Pharm Biomed Anal 30(4):1233–1247

Jones C, Lemercinier X, Crane DT, Gee CK, Austin S (2000) Spectroscopic studies of the structure and stability of glycoconjugate vaccines. Dev Biol (Basel) 103:121–136

Jones C, Ravenscroft N (2008) NMR assays for carbohydrate-based vaccines. In: Holtzgrabe U, Wawer I, Diehl B (eds) NMR spectroscopy in pharmaceutical analysis. Elsevier, Oxford

Jonson G, Osek J, Svennerholm AM, Holmgren J (1996) Immune mechanisms and protective antigens of *Vibrio cholerae* serogroup O139 as a basis for vaccine development. Infect Immun 64:3778–3785

Jumel K, Ho MM, Bolgiano B (2002) Evaluation of meningococcal C oligosaccharide conjugate vaccines by size-exclusion chromatography/multi-angle laser light scattering. Biotechnol Appl Biochem 36:219–226

Kabanova A, Adamo R, Proietti D, Berti F, Tontini M, Rappuoli R, Costantino P (2010a) Preparation, characterization and immunogenicity of HIV-1 related high-mannose oligosaccharides-CRM_{197} glycoconjugates. Glycoconj J 27:501–513. doi:10.1007/s10719-010-9295-0

Kabanova A, Margarit I, Berti F, Romano MR, Grandi G, Bensi G, Chiarot E, Proietti D, Swennen E, Cappelletti E, Fontani P, Casini D, Adamo R, Pinto V, Skibinski D, Capo S, Buffi G, Gallotta M, Christ WJ, Campbell AS, Pena J, Seeberger PH, Rappuoli R, Costantino P (2010b) Evaluation of a Group A *Streptococcus* synthetic oligosaccharide as vaccine candidate. Vaccine 29:104–114. doi:10.1016/j.vaccine.2010.09.018

Kalay H, Ambrosini M, van Berkel PH, Parren PW, van Kooyk Y, García Vallejo JJ (2012) Online nanoliquid chromatography–mass spectrometry and nanofluorescence detection for high-resolution quantitative N-glycan analysis. Anal Biochem 423(1):153–162

Kao G, Tsai CM (2004) Quantification of O-acetyl, N-acetyl and phosphate groups and determination of the extent of O-acetylation in bacterial vaccine polysaccharides by high-performance anion-exchange chromatography with conductivity detection (HPAEC-CD). Vaccine 22(3–4):335–344

Kenne L, Lindberg B, Unger P, Gustafsson B, Holme T (1982) Structural studies of the *Vibrio cholerae* O-antigen. Carbohydr Res 100:341–349

Killen KP, Griffin TJ, Cartee RT, Thanawastien A (2013) Development of protein capsular matrix vaccine platform technology. Bioprocess Int 11:S26–S32

Kilpi T, Åhman H, Jokinen J, Lankinen KS, Palmu A, Savolainen H, Gronholm M, Leinonen M, Hovi T, Eskola J, Kayhty H, Bohidar N, Sadoff JC, Finnish Otitis Medisa Study Group, Mäkelä PH (2003) Protective efficacy of a second pneumococcal conjugate vaccine against pneumococcal acute otitis media in infants and children: randomized, controlled trial of a 7-

valent pneumococcal polysaccharide-meningococcal outer membrane protein complex conjugate vaccine in 1666 children. Clin Infect Dis 37(9):1155–1164
Kim JS, Laskowich ER, Arumugham RG, Kaiser RE, MacMichael GJ (2005) Determination of saccharide content in pneumococcal polysaccharides and conjugate vaccines by GC-MSD. Anal Biochem 347(2):262–274
Kim JS, Laskowich ER, Michon F, Kaiser RE, Arumugham RG (2006a) Monitoring activation sites on polysaccharides by GC-MS. Anal Biochem 358(1):136–142
Kim JS, Reuhs BL, Michon F, Kaiser RE, Arumugham RG (2006b) Addition of glycerol for improved methylation linkage analysis of polysaccharides. Carbohydr Res 341(8):1061–1064
Klein DL, Martinez JE, Hickey MH, Hassouna F, Zaman K, Steinhoff M (2012) Development and characterization of a multiplex bead-based immunoassay to quantify pneumococcal capsular polysaccharide-specific antibodies. Clin Vaccine Immunol 19(8):1276–1282
Kniskern PJ, Ip CC, Hagopian A, Hennessey JP Jr, Miller WJ, Kubek DJ, Burke PD, Marburg S, Tolman RL (2010) Pneumococcal polysaccharide conjugate vaccine. Office E.P. 92300655.5. EP 0497525B2
Kniskern PJ, Marburg S (1994) Conjugation: design, chemistry, and analysis. In: Ellis RW, Granoff DM (eds) Development and clinical uses of *Haemophilus* b conjugate vaccines. Marcel Dekker, New York
Konadu EE, Shiloach JJ, Bryla DAD, Robbins JB, Szu SC (1996) Synthesis, characterization, and immunological properties in mice of conjugates composed of detoxified lipopolysaccharide of *Salmonella paratyphi* A bound to tetanus toxoid with emphasis on the role of O-acetyls. Infect Immun 64:2709–2715
Konadu EY, Lin FYC, Ho VA, Thuy NT, Van Bay P, Thanh TC, Khiem HB, Trach DD, Karpas AB, Li J, Bryla DA, Robbins JB, Szu SC (2000) Phase 1 and phase 2 studies of *Salmonella enterica Serovar paratyphi* A O-specific polysaccharide-tetanus toxoid conjugates in adults, teenagers, and 2- to 4-year-old children in Vietnam. Infect Immun 68:1529–1534. doi:10.1128/IAI.68.3.1529-1534.2000
Kowarik M, Ravenscroft N, Braun M, Wetter M, Haeuptle MA, Steffen M, Carranza P, Wacker M (2012) Development and analysis of a glycoconjugate vaccine against *Shigella flexneri* 2a. In: 5th Baltic meeting on microbial carbohydrates, Suzdal, Russia, September 2012
Kowarik M, Young NM, Numao S, Schulz BL, Hug I, Callewaert N, Mills DC, Watson DC, Hernandez M, Kelly JF, Wacker M, Aebi M (2006) Definition of the bacterial N-glycosylation site consensus sequence. EMBO J 25(9):1957–1966
Kubler-Kielb J, Vinogradov E, Lagergård T, Ginzberg A, King JD, Preston A, Maskell DJ, Pozsgay V, Keith JM, Robbins JB, Schneerson R (2011) Oligosaccharide conjugates of *Bordetella pertussis* and *bronchiseptica* induce bactericidal antibodies, an addition to pertussis vaccine. PNAS 108(10):4087–4092. doi:10.1073/pnas.1100782108
Laferriere C, Ravenscroft N, Wilson S, Combrink J, Gordon L, Petre J (2011) Experimental design to optimize an *Haemophilus influenzae* type b conjugate vaccine made with hydrazide-derivatized tetanus toxoid. Glycoconjugate J 28(7):463–472
Lamb DH, Lei QP, Hakim N, Rizzo S, Cash P (2005) Determination of meningococcal polysaccharides by capillary zone electrophoresis. Anal Biochem 338(2):263–269
Lamb DH, Summa L, Lei QP, Duval G, Adam O (2000) Determination of free carrier protein in protein-polysaccharide conjugate vaccines by micellar electrokinetic chromatography. J Chromatogr A 894(1–2):311–318
Lancaster L, Saydam M, Markey K, Ho MM, Mawas F (2011) Immunogenicity and physicochemical characterization of a candidate conjugate vaccine against Group B *Streptococcus* serotypes Ia, Ib and III. Vaccine 29:3213–3221. doi:10.1016/j.vaccine.2011.02.039
Laxminarayan R, Duse A, Wattal C, Zaidi AK, Wertheim HF, Sumpradit N, Vlieghe E et al (2013) Antibiotic resistance—the need for global solutions. Lancet Infect Dis 13(12):1057–1098
Lee CH, Kuo WC, Beri S, Kapre S, Joshi JS, Bouveret N, LaForce FM, Frasch CE (2009) Preparation and characterization of an immunogenic meningococcal Group A conjugate vaccine for use in Africa. Vaccine 27(5):726–732

Lee JC, Gambillara V, Alaimo C, Lipowsky G, Kowarik M, Desgrandchamps D, Wacker M (2011) Glycoprotein vaccines: a new approach to prevent *Staphylococcus aureus* infection by combining capsular polysaccharide and protein antigens. In: 51st Interscience conference on antimicrobial agents and chemotherapy, Chicago, IL, 17–20 Sept 2011

Lei QP, Lamb DH, Heller R, Pietrobon P (2000a) Quantitation of low level unconjugated polysaccharide in tetanus toxoid-conjugate vaccine by HPAEC/PAD following rapid separation by deoxycholate/HCl. J Pharm Biomed Anal 21:1087–1091

Lei QP, Lamb DH, Shannon AG, Cai X, Heller RK, Huang M, Zablackis E, Ryall R, Cash P (2004) Quantification of residual EDU (*N*-ethyl-N′-(dimethylaminopropyl)carbodiimide (EDC) hydrolyzed urea derivative) and other residual by LC-MS/MS. J Chromatogr B 813:103–112

Lei QP, Shannon AG, Heller RK, Lamb DH (2000b) Quantification of free polysaccharide in meningococcal polysaccharide-diphtheria toxoid conjugate vaccines. Dev Biol (Basel) 103:259–264

Lemercinier X, Jones C (1996) Full ^{1}H NMR assignment and detailed O-acetylation patterns of capsular polysaccharides from *Neisseria meningitidis* used in vaccine production. Carbohydr Res 296:83–96

Lemercinier X, Jones C (2000) An NMR spectroscopic identity test for the control of the capsular polysaccharide from *Haemophilus influenzae* type b. Biologicals 28:175–183

Li Y, Lander R, Manger W, Lee A (2004) Determination of lipid profile in meningococcal polysaccharide using reversed-phase liquid chromatography. J Chromatogr B 804(2):353–358

Lin FY, Ho VA, Khiem HB, Trach DD, Bay PV, Thanh TC, Kossaczka Z, Bryla DA, Shiloach J, Robbins JB, Schneerson R, Szu SC (2001) The efficacy of a *Salmonella typhi* Vi conjugate vaccine in two-to-five-year-old children. N Engl J Med 344:1263–1269. doi:10.1056/NEJM200104263441701

Lindberg AA (1999) Glycoprotein conjugate vaccines. Vaccine 17(Suppl 2):S28–S36

Lipinski T, Fitieh A, St Pierre J, Ostergaard HL, Bundle DR, Touret N (2013) Enhanced immunogenicity of a tricomponent mannan tetanus toxoid conjugate vaccine targeted to dendritic cells via Dectin-1 by incorporating β-glucan. J Immunol 190(8):4116–4128. doi:10.4049/jimmunol.1202937

Lipinski T, Wu X, Sadowska J, Kreiter E, Yasui Y, Cheriaparambil S, Rennie R, Bundle DR (2012) A β-mannan trisaccharide conjugate vaccine aids clearance of *Candida albicans* in immunocompromised rabbits. Vaccine 30(44):6263–6269. doi:10.1016/j.vaccine.2012.08.010

Losonsky GA, Lim Y, Motamedi P, Comstock LE, Johnson JA, Morris JG Jr, Tacket CO, Kaper JB, Levine MM (1997) Vibriocidal antibody responses in North American volunteers exposed to wild-type or vaccine *Vibrio cholerae* O139: specificity and relevance to immunity. Clin Diagn Lab Immunol 4:264–269

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193(1):265–275

Lundborg M, Widmalm G (2011) Structure analysis of glycans by NMR chemical shift prediction. Anal Chem 83:1514–1517. doi:10.1021/ac1032534

Lyngby J, Olsen LH, Eidem T, Lundanes E, Jantzen E (2002) Quantification of lipopolysaccharides in outer membrane vesicle vaccines against meningococcal disease. High-performance liquid chromatographic determination of the constituent 3-hydroxy-lauric acid. Biologicals 30(1):7–13

MacLennan CM, Martin LB, Micoli M (2014) Vaccines against invasive *Salmonella* diseases: current status and future directions. Hum Vaccine Immunother 10(6):1–16

MacLeod CM, Hodges RG, Heidelberger M, Bernhard WG (1945) Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides. J Exp Med 82:445–465

MacNair JE, Desai T, Teyral J, Abeygunawardana C, Hennessey JP Jr (2005) Alignment of absolute and relative molecular size specifications for a polyvalent pneumococcal polysaccharide vaccine (PNEUMOVAX®23). Biologicals 33:49–58

Madhi SA, Dangor Z, Heath PT, Schrag S, Izu A, Sobanjo-ter Meulen A, Dull PM (2013). Considerations for a phase-III trial to evaluate a Group B *Streptococcus* polysaccharide-protein conjugate vaccine in pregnant women for the prevention of early-and late-onset invasive disease in young-infants. Vaccine 31:D52–D57

Malito E, Bursulaya B, Chen C, Lo Surdo P, Picchianti M, Balducci E, Biancucci M, Brock A, Berti F, Bottomley MJ, Nissum M, Costantino P, Rappuoli R, Spraggon G (2012) Structural basis for lack of toxicity of the diphtheria toxin mutant CRM_{197}. Proc Natl Acad Sci USA 109 (14):5229–5234

Malito E, Faleri A, Lo Surdo P, Veggi D, Maruggi G, Grassi E, Cartocci E, Bertoldi I, Genovese A, Santini L, Romagnoli G, Borgogni E, Brier S, Lo Passo C, Domina M, Castellino F, Felici F, van der Veen S, Johnson S, Lea SM, Tang CM, Pizza M, Savino S, Norais N, Rappuoli R, Bottomley MJ, Masignani V (2013) Defining a protective epitope on factor H binding protein, a key meningococcal virulence factor and vaccine antigen. Proc Natl Acad Sci USA 110 (9):3304–3309. doi:10.1073/pnas.1222845110

Malkiel S, Liao L, Cunningham MW, Diamond B (2000) T-Cell-dependent antibody response to the dominant epitope of streptococcal polysaccharide, *N*-acetyl-glucosamine, is cross-reactive with cardiac myosin. Infect Immun 68:5803–5808

Marburg S, Jorn D, Tolman RL, Arison B, McCauley J, Kniskern PJ, Hagopian A, Vella PP (1986) Bimolecular chemistry of macromolecules: synthesis of bacterial polysaccharide conjugates with *Neisseria meningitidis* membrane protein. J Am Chem Soc 108:5282–5287

Marburg S, Tolman RL, Kniskern PJ, Miller WJ, Hagopian A, Ip CC, Hennessey JP Jr, Kubek DJ, Burke PD (1997) US Patent 5,623,057. U.S. Patent and Trademark Office, Washington, DC, 22 April 1997

Martin LB (2013) Development of a conjugate vaccines for enteric fever. In: 8th International conference of typhoid fever and other invasive *Salmonelloses*, Dhaka, Bangladesh, March 2013

Martin CE, Broecker F, Oberli MA, Komor J, Mattner J, Anish C, Seeberger PH (2013) Immunological evaluation of a synthetic *Clostridium difficile* oligosaccharide conjugate vaccine candidate and identification of a minimal epitope. J Am Chem Soc 135:9713–9722. doi:10.1021/ja401410y

Matsumoto T, Nieuwenhuis EE, Cisneros RL, Ruiz-Perez B, Yamaguchi K, Blumberg RS, Onderdonk AB (2004) Protective effect of ethyl-3-(3-dimethyl aminopropyl) urea dihydrochloride (EDU) against LPS-induced death in mice. J Med Microbiol 53(2):97–102

McCarthy PC, Saksena R, Peterson DC, Lee CH, An Y, Cipollo JF, Vann WF (2013) Chemoenzymatic synthesis of immunogenic meningococcal Group C polysialic acid-tetanus Hc fragment glycoconjugates. Glycoconj J 30:857–870. doi:10.1007/s10719-013-9490-x

McLellan JS, Pancera M, Carrico C, Gorman J, Julien JP, Khayat R, Louder R, Pejchal R, Sastry M, Dai K, O'Dell S, Patel N, Shahzad-ul-Hussan S, Yang Y, Zhang B, Zhou T, Zhu J, Boyington JC, Chuang GY, Diwanji D, Georgiev I, Kwon YD, Lee D, Louder MK, Moquin S, Schmidt SD, Yang ZY, Bonsignori M, Crump JA, Kapiga SH, Sam NE, Haynes BF, Burton DR, Koff WC, Walker LM, Phogat S, Wyatt R, Orwenyo J, Wang LX, Arthos J, Bewley CA, Mascola JR, Nabel GJ, Schief WR, Ward AB, Wilson IA, Kwong PD (2011) Structure of HIV-1 gp120 V1/V2 domain with broadly neutralizing antibody PG9. Nature 480(7377):336–343. doi:10.1038/nature10696

McMaster R (2000) Purification of polysaccharide-protein conjugate vaccines by ultrafiltration with ammonium sulfate solutions. US Patent 6,146,902, 14 Nov 2000

McNeely TB, Staub JM, Rusk CM, Blum MJ, Donnelly JJ (1998) Antibody responses to capsular polysaccharide backbone and O-acetate side groups of *Streptococcus pneumoniae* type 9V in humans and rhesus macaques. Infect Immun 66(8):3705–3710

Meeks MD, Saksena R, Ma X, Wade TK, Taylor RK, Kovac P, Wade WF (2004) Synthetic fragments of *Vibrio cholerae* O1 Inaba O-specific polysaccharide bound to a protein carrier are immunogenic in mice but do not induce protective antibodies. Infect Immun 72:4090–4101. doi:10.1128/IAI.72.7.4090-4101.2004

Metz B, Hendriksen CF, Jiskoot W, Kersten GF (2002) Reduction of animal use in human vaccine quality control: opportunities and problems. Vaccine 20:2411–2430

Metz B, Tilstra W, van der Put R, Spruit N, van den IJssel J, Robert J, Hendrikson C, Kersten G (2013) Physicochemical and immunochemical assays for monitoring consistent production of tetanus toxoid. Biologicals 41(4):231–237

Michon F, Moore SL, Kim J, Blake MS, Auzanneau FI, Johnston BD, Johnson MA, Pinto BM (2005) Doubly branched hexasaccharide epitope on the cell wall polysaccharide of group A streptococci recognized by human and rabbit antisera. Infect Immun 73:6383–6389. doi:10.1128/IAI.73.10.6383-6389.2005

Micoli F, Ravenscroft N, Cescutti P, Stefanetti G, Londero S, Rondini S, MacLennan CA (2014) Structural analysis of O-polysaccharide chains extracted from different *Salmonella typhimurium* strains. Carbohydr Res 385:1–8

Micoli F, Romano MR, Tontini M, Cappelletti E, Massimiliano G, Proietti D, Rondini S, Swennen E, Santini L, Filippini S, Balochi C, Adamo R, Pluschke G, Norheim G, Pollard A, Saul A, Rappuoli R, MacLennan CA, Berti F, Costantino P (2013a) Development of a glycoconjugate vaccine to prevent meningitis in Africa caused by meningococcal serogroup X. Proc Natl Acad Sci USA 110(47):19077–19082. doi:10.1073/pnas.1314476110

Micoli F, Rondini S, Gavini M, Pisoni I, Lanzilao L, Colucci AM, Giannelli AM, Pippi F, Sollai L, Pinto V, Berti F, MacLennan CA, Martin LB, Saul A (2013b) A scalable method for O-antigen purification applied to various *Salmonella serovars*. Anal Biochem 434:136–145. doi:10.1016/j.ab.2012.10.038

Micoli F, Rondini S, Pisoni I, Giannelli C, Di Cioccio V, Costantion P, Saul A, Martin LB (2012) Production of a conjugate vaccine for *Salmonella enterica Serovar typhi* from *Citrobacter* Vi. Vaccine 30:853–861. doi:10.1016/j.vaccine.2011.11.108

Micoli F, Rondini S, Pisoni I, Proietti D, Berti F, Costantino P, Rappuoli R, Szu S, Saul A, Martin LB (2011a) Vi-CRM_{197} as a new conjugate vaccine against *Salmonella typhi*. Vaccine 29 (4):712–720. doi:10.1016/j.vaccine.2010.11.022

Micoli F, Rondini S, Gavini M, Lanzilao L, Medaglini D, Saul A, Martin LB (2011b) O:2-CRM_{197} conjugates against *Salmonella paratyphi* A. PLoS ONE 7:e47039–e47039. doi:10.1371/journal.pone.0047039

Mistretta N, Danve E, Moreau M (2010) Conjugates obtained by reductive amination of the pneumococcus serotype 5 capsular polysaccharide. US Patent 7,812,006, 12 Oct 2010

Mittermayr S, Bones J, Guttman A (2013) Unraveling the glyco-puzzle: glycan structure identification by capillary electrophoresis. Anal Chem 85(9):4228–4238

Monteiro MA, Ma Z, Bertolo L, Jiao Y, Arroyo L, Hodgins D, Mallozzi M, Vedantam G, Sagermann M, Sundsmo J, Chow H (2013) Carbohydrate-based *Clostridium difficile* vaccines. Expert Rev Vaccines 12:421–431. doi:10.1586/erv.13.9

Morelli L, Lay L (2013) Synthesis of *Neisseria meningitidis* X capsular polysaccharide fragments. ARKIVOC (ii):166–184

Morelli L, Cancogni D, Tontini M, Nilo A, Filippini S, Costantino P, Romano MR, Berti F, Adamo R, Lay L (2014) Synthesis and Immunological Evaluation of Protein Conjugates of Neisseria meningitidis X Capsular Polysaccharide Fragments. Beilstein J Org Chem 2014 (in press)

Mosley SL, Rancy PC, Peterson DC, Vionnet J, Saksena R, Vann WF (2010) Chemoenzymatic synthesis of conjugatable oligosialic acids. Biocatal Biotransform 28:41–50. doi:10.3109/10242420903388694

Muindi KM, McCarthy PC, Wang T, Vionnet J, Jankowska E, Vann WF (2013) Characterization of the meningococcal serogroup X capsule *N*-acetylglucosamine-1-phosphotransferase. Glycobiology 24(2):139–149. doi:10.1093/glycob/cwt091

Mukherjee J, Nussbaum G, Scharff MD, Casadevall A (1995) Protective and nonprotective monoclonal antibodies to *Cryptococcus neoformans* originating from one B cell. J Exp Med 181:405–409

Munoz EC, Baldor CT, Munoz ME, Cabrera RA, Infante JFB, Sierra GG (2000) Identification, interaction and Kd studies of bacterial biopolymers by use of superose 6 gels and UV monitoring. Acta Chromatographica 10:147–156

Nakouzi A, Zhang T, Oscarson S, Casadevall A (2009) The common *Cryptococcus neoformans* glucuronoxylomannan M2 motif elicits non-protective antibodies. Vaccine 27:3513–3518. doi:10.1016/j.vaccine.2009.03.089

Ni J, Song H, Wang Y, Stamatos NM, Wang LX (2006) Toward a carbohydrate-based HIV-1 vaccine: synthesis and immunological studies of oligomannose-containing glycoconjugates. Bioconjugate Chem 17:493–500. doi:10.1021/bc0502816

Nitz M, Ling C-C, Otter A, Cutler JE, Bundle DR (2002) The unique solution structure and immunochemistry of the *Candida albicans* beta-1,2-mannopyranan cell wall antigens. J Biol Chem 277:3440–3446. doi:10.1074/jbc.M109274200

Noe SM, Green MA, HogenEsch H, Hem SL (2010) Mechanism of immunopotentiation by aluminum-containing adjuvants elucidated by the relationship between antigen retention at the inoculation site and the immune response. Vaccine 28(20):3588–3594

Nurkka A, Åhman H, Yaich M, Eskola J, Käyhty H (2001) Serum and salivary anti-capsular antibodies in infants and children vaccinated with octavalent pneumococcal conjugate vaccines, PncD and PncT. Vaccine 20(1):194–201

Oberli MA, Hecht ML, Bindschädler P, Adibekian A, Adam T, Seeberger PH (2011) A possible oligosaccharide-conjugate vaccine candidate for *Clostridium difficile* is antigenic and immunogenic. Chem Biol 18(5):580–588. doi:10.1016/j.chembiol.2011.03.009

Oliver MB, Jones C, Larson TR, Calix JJ, Zartler ER, Yother J, Nahm MH (2013) *Streptococcus pneumoniae* serotype 11D has a bi-specific glycosyltransferase and expresses two different capsular polysaccharide repeating units. J Biol Chem 288(30):21945–21954. doi:10.1074/jbc.M113.488528

Oscarson S, Alpe M, Svahnberg P, Nakouzi A, Casadevall A (2005) Synthesis and immunological studies of glycoconjugates of *Cryptococcus neoformans* capsular glucuronoxylomannan oligosaccharide structures. Vaccine 23:3961–3972. doi:10.1016/j.vaccine.2005.02.029

Paoletti LC, Kasper DL (2003) Glycoconjugate vaccines to prevent group B streptococcal infections. Expert Opin Biol Ther 3:975–984. doi:10.1517/14712598.3.6.975

Paoletti LC, Kasper DL, Michon F (1992a) Effects of chain length on the immunogenicity in rabbits of group B *Streptococcus* type III oligosaccharide-tetanus toxoid conjugates. J Clin Invest 89(1):203–209

Paoletti LC, Kasper DL, Michon F, DiFabio J, Holme K, Jennings HJ, Wessels MR (1990) An oligosaccharide-tetanus toxoid conjugate vaccine against type III group B *Streptococcus*. J Biol Chem 265(30):18278–18283

Paoletti LC, Wessels MR, Michon F, DiFabio J, Jennings HJ, Kasper DL (1992b) Group B *Streptococcus* type II polysaccharide-tetanus toxoid conjugate vaccine. Infect Immun 60 (10):4009–4014

Park IH, Pritchard DG, Cartee R, Brandao A, Brandileone MCC, Nahm MH (2007) Discovery of a new capsular serotype (6C) within serogroup 6 of *Streptococcus pneumoniae*. J Clin Micro 45 (4):1225–1233. doi:10.1128/IAI.00510-07

Park JT, Johnson MJ (1949) A submicrodetermination of glucose. J Biol Chem 181:149–151

Peeters CC, Tenbergen-Meekes AMJ, Poolman JT, Zegers BJ, Rijkers GT (1992) Immunogenicity of a *Streptococcus pneumoniae* type 4 polysaccharide-protein conjugate vaccine is decreased by admixture of high doses of free saccharide. Vaccine 10(12):833–840

Pejchal R, Doores KJ, Walker LM, Khayat R, Huang PS, Wang SK, Stanfield RL, Julien JP, Ramos A, Crispin M, Depetris R, Katpally U, Marozsan A, Cupo A, Maloveste S, Liu Y, McBride R, Ito Y, Sanders RW, Ogohara C, Paulson JC, Feizi T, Scanlan CN, Wong CH, Moore JP, Olson WC, Ward AB, Poignard P, Schief WR, Burton DR, Wilson IA (2011) A potent and broad neutralizing antibody recognizes and penetrates the HIV glycan shield. Science 334(6059):1097–1103. doi:10.1126/science.1213256

Peltola H, Käythy H, Sivonen A, Mäkelä PH (1977) *Haemophilus influenzae* type b capsular polysaccharide vaccine in children: a double-blind field study of 100,000 vaccinees 3 months to 5 years of age in Finland. Pediatrics 60(5):730–737

Petersen B, Hindsgaul O, Meier S (2013) Profiling of carbohydrate mixtures at unprecedented resolution using high-precision ^{1}H-^{13}C chemical shift measurements and a reference library. Analyst. doi:10.1039/C3AN01922E

Phalipon A, Mulard LA, Sansonetti PJ (2008) Vaccination against shigellosis: is it the path that is difficult or is it the difficult that is the path? Microbes Infect 10:1057–1062. doi:10.1016/j.micinf.2008.07.016

Phalipon A, Tanguy M, Grandjean C, Guerreiro C, Belot F, Cohen D, Sansonetti PJ, Mulard LA (2009) A synthetic carbohydrate-protein conjugate vaccine candidate against *Shigella flexneri* 2a infection. J Immunol 182:2241–2247. doi:10.4049/jimmunol.0803141

Pinto V, Berti F (2014) Exploring the Group B *Streptococcus* capsular polysaccharides: the structural diversity provides the basis for development of NMR-based identity assays. J Pharm Biomed Anal 98:9–15

Pittman M (1931) Variation and type specificity in the bacterial species *Haemophilus influenzae*. J Exp Med 53:471–492

Podda A (2010) Aims and role of Novartis Vaccines Institute for Global Health (NVGH). Procedia Vaccinol 2(2):124–127

Rohrer JS, Basumallick L, Hurum D (2013) High-performance anion-exchange chromatography with pulsed amperometric detection for carbohydrate analysis of glycoproteins. Biochem (Moscow) 78(7):697–709

Pon RA (2012) Exploiting the bacterial surface: the successful application of glycoconjugate vaccines. In: Reid CW, Twine SM, Reid AN (eds) Bacterial glycomics: current research, technology, and applications. Academic Press, Norfolk

Poolman J, Borrow R (2011) Hyporesponsiveness and its clinical implications after vaccination with polysaccharide or glycoconjugate vaccines. Expert Rev Vaccines 10(3):307–322

Porro M (1994) Oligosaccharide conjugate vaccines US Patent 5,306,492. U.S. Patent and Trademark Office, Washington, DC

Pozzi C, Wilk K, Lee JC, Gening M, Nifantiev N, Pier GB (2012) Opsonic and protective properties of antibodies raised to conjugate vaccines targeting six *Staphylococcus aureus* antigens. PLoS ONE 7:e46648. doi:10.1371/journal.pone.0046648

Prasad AK (2011) Multivalent pneumococcal polysaccharide-protein conjugate composition. US Patent 7,955,605. U.S. Patent and Trademark Office, Washington, DC

Proietti D, Abballe F, Bardotti A, Ricci S, D'Ascenzi S (2005) Chromatographic characterization of bacterial oligosaccharides by anion exchange profiles combining different detection techniques. Glycoconj J 22(4–6):232 (Abstract P001 from GLYCO XVIII, the XVIII international symposium on glycoconjugates, September 4–9, 2005, Firenze, Italy)

Prymula R, Peeters P, Chrobok V, Kriz P, Novakova E, Kaliskova E, Kohl I, Lommel P, Poolman J, Prieels JP, Schuerman L (2006) Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both *Streptococcus pneumoniae* and non-typable *Haemophilus influenzae*: a randomised double-blind efficacy study. Lancet 367:740–748

Pujar NS, Huang NF, Daniels CL, Dieter L, Gayton MG, Lee AL (2004) Base hydrolysis of phosphodiester bonds in pneumococcal polysaccharides. Biopolymers 75(1):71–84

Qadri F, Ahmed F, Karim MM, Wenneras C, Begum YA, Abdus Salam M, Albert MJ, McGhee JR (1999) Lipopolysaccharide- and cholera toxin-specific subclass distribution of B-cell responses in cholera. Clin Diagn Lab Immunol 6:812–818

Qi XY, Keyhani NO, Lee YC (1988) Spectroscopic determination of hydrazine, hydrazides, and their mixtures with trinitrobenzenesulfonic acid. Anal Biochem 175:139–144

Rahman MM, Guard-Petter J, Carlson RW (1997) A virulent isolate of *Salmonella enteritidis* produces a *Salmonella typhi*-like lipopolysaccharide. J Bacteriol 179:2126–2131

Ravenscroft N (2000) The application of NMR spectroscopy to track the industrial preparation of polysaccharide and derived glycoconjugate vaccines. Pharmeuropa, Special Edition, pp 131–144

Ravenscroft N, Averani G, Bartoloni A, Berti S, Bigio M, Carinci V, Costantino P, D'Ascenzi S, Giannozzi A, Norelli F, Pennatini C, Proietti D, Ceccarini C, Cescutti P (1999) Size determination of bacterial capsular oligosaccharides used to prepare conjugate vaccines. Vaccine 17:2802–2816

Ravenscroft N, D'Ascenzi S, Proietti D, Norelli F, Costantino P (2000) Physicochemical characterisation of the oligosaccharide component of vaccines. Dev Biol (Basel) 103:35–47

Ravenscroft N, Feavers IM (2006) Conjugate vaccines. In: Frosch M, Maiden M (eds) Meningococcal disease. Pathogenicity and prevention. Wiley-VCH, Weinheim

Ravenscroft N, Wheeler JX, Jones C (2010) Bioanalysis of meningococcal vaccines. Bioanalysis 2 (2):343–361

Ravenscroft N, Fernandez F, Braun M, Wetter M, Haeuptle MA, Steffen M, Carranza P, Gambillara V, Kowarik M, Wacker M (2012) Development and analysis of a glycoconjugate vaccine against *Shigellosis*. In: 26th International carbohydrate symposium. Madrid, July 2012

Reid CW, Vinogradov E, Li J, Jarrell HC, Logan SM, Brisson JR (2012) Structural characterization of surface glycans from *Clostridium difficile*. Carbohydr Res 354:65–73. doi:10.1016/j.carres.2012.02.002

Ricci S, Bardotti A, D'Ascenzi S, Ravenscroft N (2001) Development of a new method for the quantitative analysis of extracellular polysaccharide of *Neisseria meningitidis* serogroup A by use of high-performance anion-exchange chromatography with pulsed-amperometric detection. Vaccine 19:1989–1997

Robbins JR, Schneerson R, Pittman M (1984) *Haemophilus influenzae* type b infectlons. In: Germanier R (ed) Bacterial vaccines. Academic Press, New York

Rodriguez ME, van den Dobbelsteen GP, Oomen LA, de Weers O, van Buren L, Beurret M, Poolman JT, Hoogerhout P (1998) Immunogenicity of *Streptococcus pneumoniae* type 6B and 14 polysaccharide-tetanus toxoid conjugates and the effect of uncoupled polysaccharide on the antigen-specific immune response. Vaccine 16(20):1941–1949

Romano MR, Leuzzi R, Cappelletti E, Tontini M, Nilo A, Proietti D, Berti F, Costantino P, Adamo R, Scarselli M (2014) Recombinant *Clostridium difficile* toxin fragments as carrier protein for PSII surface polysaccharide preserve their neutralizing activity. Toxins 6(4):1385–1396. doi:10.3390/toxins6041385

Romanow A, Haselhorst T, Stummeyer K, Claus H, Bethe A, Muhlenhoff M, Vogel U, von Itzstein M, Gerardy-Schahn R (2013) Biochemical and biophysical characterization of the sialyl/hexosyl-transferase synthesizing the meningococcal serogroup W135 heteropolysaccharide capsule. J Biol Chem 288(17):11718–11730. doi:10.1074/jbc.M113.452276

Ruiz-Perez B, Cisneros RL, Matsumoto T, Miller RJ, Vasios G, Calias P, Onderdonk AB (2003) Protection against lethal intra-abdominal sepsis by 1-(3-dimethylaminopropyl)-3-ethylurea. J Infect Dis 188(3):378–387

Ruttens B, Kováč P (2006) Synthesis of spacer-equipped phosphorylated di-, tri- and tetrasaccharide fragments of the O-specific polysaccharide of *Vibrio cholerae* O139. Carbohydr Res 341:1077–1080. doi:10.1016/j.carres.2006.04.007

Sabharwal H, Michon F, Nelson D, Dong W, Fuchs K, Manjarrez RC, Sarkar A, Uitz C, Viteri-Jackson A, Suarez RSR, Blake M, Zabriskie JB (2006) Group A *Streptococcus* (GAS) carbohydrate as an immunogen for protection against GAS infection. J Infect Dis 193:129–135. doi:10.1086/498618

Saksena R, Ma X, Wade TK, Kovac P, Wade WF (2005) Effect of saccharide length on the immunogenicity of neoglycoconjugates from synthetic fragments of the O-SP of *Vibrio cholerae* O1, serotype Ogawa. Carbohydr Res 340(14):2256–2269. doi:10.1016/j.carres.2005.07.017

Saksena R, Ma X, Wade TK, Kovac P, Wade WF (2006) Length of the linker and the interval between immunizations influences the efficacy of *Vibrio cholerae* O1, Ogawa hexasaccharide neoglycoconjugates. FEMS Immunol Med Microbiol 47(1):116–128. doi:10.1111/j.1574-695X.2006.00071.x

Salvadori LG, Blake MS, McCarty M, Tai JY, Zabriskia JB (1995) Group A streptococcus-liposome ELISA antibody titers to group A polysaccharide and opsonophagocytic capabilities of the antibodies. J Infect Dis 171:593–600

Santosham M, Wolff M, Reid R, Hohenboken M, Bateman M, Goepp J et al (1991) The efficacy in Navajo infants of a conjugate vaccine consisting of *Haemophilus influenzae* type b polysaccharide and *Neisseria meningitidis* outer-membrane protein complex. N Engl J Med 324:1767–1772

Sawicki E, Hauser TR, Stanley TW, Elbert W (1961) The 3-methyl-2-benzothiazolone hydrazone test: sensitive new methods for the detection, rapid estimation, and determination of aliphatic aldehydes. Anal Chem 33:93–96

Schneerson R, Barrera O, Sutton A, Robbins JB (1980) Preparation, characterization and immunogenicity of *Haemophilus influenzae* type b polysaccharide-protein conjugates. J Exp Med 152:361–376

Schuchat A (1998) Epidemiology of group B streptococcal disease in the United States: shifting paradigms. Clin Microbiol Rev 11:497–513

Schulz D, Rapp P (1991) Properties of the polyalcohol prepared from the β-d-glucan schizophyllan by periodate oxidation and borohydride reduction. Carbohydr Res 222:223–231

Seid RC Jr, Boykins RA, Liu DF, Kimbrough KW, Hsieh CL, Eby R (1989) Chemical evidence for covalent linkages of a semisynthetic glycoconjugate vaccine for *Haemophilus influenzae* type b disease. Glycoconjugate J 6(4):489–497

Schafer DE, Toll B, Schuman RF, Nelson BL, Mond JJ, Lees A (2000) Activation of soluble polysaccharides with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) for use in protein polysaccharide conjugate vaccines and immunological reagents. II. Selective crosslinking of proteins to CDAP-activated polysaccharides. Vaccine 18:1273–1281

Shikhman AR, Greenspan NS, Cunningham MW (1993) A subset of mouse monoclonal antibodies cross-reactive with cytoskeletal proteins and group A streptococcal M proteins recognizes N-acetyl-beta-D-glucosamine. J Immunol 151:3902–3913

Shinefield H, Black S, Fattom A, Horwith G, Rasgon S, Ordonez J, Yeoh H, Law D, Robbins JB, Schneerson R, Muenz L, Fuller S, Johnson J, Fireman B, Alcorn H, Naso R (2002) Use of a *Staphylococcus aureus* conjugate vaccine in patients receiving hemodialysis. N Engl J Med 346:491–496. doi:10.1056/NEJMoa011297

Shinefield HR (2010) Overview of the development and current use of CRM_{197} conjugate vaccines for pediatric use. Vaccine 28(27):4335–4339

Silveira IA, Bastos RC, Neto MS, Laranjeira AP, Assis EF, Fernandes SA, Leal ML, Silva WC, Lee CH, Frasch CE, Peralta JM, Jessouroun E (2007) Characterization and immunogenicity of meningococcal group C conjugate vaccine prepared using hydrazide-activated tetanus toxoid. Vaccine 25(41):7261–7270

Simon R, Tennant MS, Wang JY, Schmidlein PJ, Lees A, Ernst RK, Pasetti MF, Galen JE, Levine MM (2011) *Salmonella enterica Serovar enteritidis* core O polysaccharide conjugated to H:g, m Flagellin as a candidate vaccine for protection against invasive infection with *S. enteritidis*. Infect Immun 79(10):4240–4249

Simon R, Levine MM (2012) Glycoconjugate vaccine strategies for protection against invasive *Salmonella* infections. Hum Vaccin Immunother 8(4):494–498

Simon S, Wang JY, Boyd MA, Tulapurkar ME, Ramachandran G, Tennant SM, Pasetti M, Galen JE, Levine MM (2013) Sustained protection in mice immunized with fractional doses of *Salmonella enteritidis* core and O polysaccharide-flagellin glycoconjugates. PLoS ONE 8(4):1–4

Skinner JM, Indrawati L, Cannon J, Blue J, Winters M, Macnair J, Pujar N, Manger W, Zhang Y, Antonello J, Shover J, Caulfied M, Heinrichs JH (2011) Pre-clinical evaluation of a 15-valent pneumococcal conjugate vaccine (PCV15-CRM197) in an infant-rhesus monkey immunogenicity model. Vaccine 29:8870–8876. doi:10.1016/j.vaccine.2011.09.078

Skoff TH, Farley MM, Petit S, Craig AS, Schaffner W, Gershman K, Harrison LH, Lynfield R, Mohle-Boetani J, Zansky S, Albanese BA, Stefonek K, Zell ER, Jackson D, Thompson T, Schrag SJ (2009) Increasing burden of invasive group B streptococcal disease in nonpregnant adults, 1990-2007. Clin Infect Dis 49:85–92. doi:10.1086/599369

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano M, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150(1):76–85

Snellings NJ, Johnson EM, Kopecko DJ, Collins HH, Baron LS (1981) Genetic regulation of variable Vi antigen expression in a strain of *Citrobacter freundii*. J Bacteriol 145:1010–1017

Snyder SL, Sobocinski PZ (1975) An improved 2,4,6-trinitrobenzenesulfonic acid method for the determination of amines. Anal Biochem 64:284–288

Steer AC, Law I, Matatolu L, Beall BW, Carapetis JR (2009) Global *emm* type distribution of group A streptococci: systematic review and implications for vaccine development. Lancet Infect Dis 9:611–616. doi:10.1016/S1473-3099(09)70178-1

Stollerman GH, Dale JB (2008) The importance of the group A *Streptococcus* capsule in the pathogenesis of human infections: a historical perspective. Clin Infect Dis 46:1038–1045. doi:10.1086/529194

Stone AL, Szu SC (1988) Application of optical properties of the Vi capsular polysaccharide for quantitation of the Vi antigen in vaccines for typhoid fever. J Clin Microbiol 26:719–725

Sturgess AW, Rush K, Charbonneau RJ, Lee JI, West DJ, Sitrin RD, Hennessy JP Jr (1999) *Haemophilus influenzae* type b conjugate vaccine stability: catalytic depolymerization of PRP in the presence of aluminium hydroxide. Vaccine 17:1169–1178

Suker J, Feavers IM, Corbel MJ, Jones C, Bolgiano B (2004) Control and lot release of meningococcal group C conjugate vaccines. Expert Rev Vaccines 3(5):533–540

Svennerholm L (1957) Quantitative estimation of sialic acid: II. a colorimetric resorcinol-hydrochloric acid method. Biochim Biophys Acta 24:604–611

Svennerholm AM, Holmgren J (1976) Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin/toxoid. Infect Immun 13:735–740

Sweeney JA, Sumner JS, Hennessey JP Jr (2000) Simultaneous evaluation of molecular size and antigenic stability of PNEUMOVAX 23, a multivalent pneumococcal polysaccharide vaccine. Dev Biol (Basel) 103:11–26

Szu SC, Stone AL, Robbins JD, Schneerson R, Robbins JB (1987) Vi capsular polysaccharide protein conjugates for prevention of typhoid fever—preparation, characterization and immunogenicity in laboratory animals. J Exp Med 166:1510–1524

Talaga P, Bellamy L, Moreau M (2001) Quantitative determination of C-polysaccharide in *Streptococcus pneumoniae* capsular polysaccharides by use of high-performance anion-exchange chromatography with pulsed amperometric detection. Vaccine 19:2987–2994

Talaga P, Vialle S, Moreau M (2002) Development of a high-performance anion-exchange chromatography with pulsed-amperometric detection based quantification assay for pneumococcal polysaccharides and conjugates. Vaccine 20:2474–2484

Taylor DN, Trofa AC, Sadoff J, Chu C, Bryla D, Shiloach J, Cohen D, Ashkenazi S, Lerman Y, Egan W (1993) Synthesis, characterization, and clinical evaluation of conjugate vaccines composed of the O-specific polysaccharides of *Shigella dysenteriae* type 1, *Shigella flexneri* type 2a, and *Shigella sonnei* (*Plesiomonas shigelloides*) bound to bacterial toxoids. Infect Immun 61(9):3678–3687

Teodorović P, Slättegård R, Oscarson S (2006) Synthesis of stable C-phosphonate analogues of *Neisseria meningitidis* group A capsular polysaccharide structures using modified Mitsunobu reaction conditions. Org Biomol Chem 4(24):4485–4490

Tian J-H, Fuhrmann SR, Kluepfel-Stahl S, Carmen RJ, Ellingsworth L, Flyer DC (2012) A novel fusion protein containing the receptor binding domains of *C. difficile* toxin A and toxin B elicits protective immunity against lethal toxin and spore challenge in preclinical efficacy models. Vaccine 30:4249–4258. doi:10.1016/j.vaccine.2012.04.045

Tomei M, Pacella G, Campa C, D'Ascenzi S (2009) Determination of size distributions of meningococcal oligosaccharides. P142. In: 8th Carbohydrate bioengineering meeting, Ischia, Italy, 10–13 May 2009

Torosantucci A, Bromuro C, Chiani P, De Bernardis F, Berti F, Galli C, Norelli F, Bellucci C, Polonelli L, Costantino P, Rappuoli R, Cassone A (2005) A novel glyco-conjugate vaccine against fungal pathogens. J Exp Med 202:597–606. doi:10.1084/jem.20050749

Torres-Sanchez MI, Zaccaria C, Buzzi B, Miglio G, Lombardi G, Polito L, Russo G, Lay L (2007) Synthesis and biological evaluation of phosphono analogues of capsular polysaccharide fragments from *Neisseria meningitidis* A. Chem-A Eur J 13(23):6623–6635

Trkola A, Purtscher M, Muster T, Ballaun C, Buchacher A, Sullivan N, Srinivasan K, Sodroski J, Moore JP, Katinger H (1996) Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J Virol 70:1100–1108

Tsai CM, Gu XX, Byrd RA (1994) Quantification of polysaccharide in *Haemophilus influenzae* type b conjugate and polysaccharide vaccines by high-performance anion-exchange chromatography with pulsed amperometric detection. Vaccine 12(8):700–706

Turek D, Sundgren A, Lahmann M, Oscarson S (2006) Synthesis of oligosaccharides corresponding to *Vibrio cholerae* O139 polysaccharide structures containing dideoxy sugars and a cyclic phosphate. Org Biomol Chem 4:1236–1241. doi:10.1039/b518125a

Turula V, Walters S, Singh S (2008) Arumugham R (2008) Automated colorimetric polysaccharide assays and includes preparing saccharide standards and diluted polysaccharide test samples. Wyeth, John, and Brother Ltd, USA (PCT Int Appl)

Turula VE, Kim J, Michon F, Pankratz J, Zhang Y, Yoo C (2004) An integrity assay for a meningococcal type B conjugate vaccine. Anal Biochem 327(2):261–270

Van Damme P, Kafeja F, Anemona A, Basile V, Hilbert AK, De Coster I, Rondini S, Micoli F, Khan RMQ, Marchetti E, Ci Cioccio V, Saul A, Martin LB, Podda A (2011) Safety, immunogenicity and dose ranging of a new Vi-CRM197 conjugate vaccine against typhoid fever: randomized clinical testing in healthy adults. PLoS ONE 6(9):e25398. doi:10.1371/journal.pone.0025398

Verez-Bencomo V, Fernández-Santana V, Hardy E, Toledo ME, Rodríguez MC, Heynngnezz L, Rodriguez A, Baly A, Herrera L, Izquierdo M, Villar A, Valdés Y, Cosme K, Deler ML, Montane M, Garcia E, Ramos A, Aguilar A, Medina E, Toraño G, Sosa I, Hernandez I, Martínez R, Muzachio A, Carmenates A, Costa L, Cardoso F, Campa C, Diaz M, Roy R (2004) A synthetic conjugate polysaccharide vaccine against *Haemophilus influenzae* type b. Science 305:522–525

Vinh H, Nhu NT, Nga TV, Duy PT, Campbell JI, Hoang NV, Boni MF, My PV, Parry C, Nga TT, Van Minh P, Thuy CT, Diep TS, Phuong le T, Chinh MT, Loan HT, Tham NT, Lanh MN, Mong BL, Anh VT, Bay PV, Chau NV, Farrar J, Baker S (2009) A changing picture of shigellosis in southern Vietnam: shifting species dominance, antimicrobial susceptibility and clinical presentation. BMC Infect Dis 9:204. doi:10.1186/1471-2334-9-204

Vipond C, Suker J, Jones C, Tang C, Feavers IM, Wheeler JX (2006) Proteomic analysis of a meningococcal outer membrane vesicle vaccine prepared from the group B strain NZ98/254. Proteomics 6(11):3400–3413

Von Hunolstein C, Parisi L, Bottaro D (2003) Simple and rapid technique for monitoring the quality of meningococcal polysaccharides by high performance size-exclusion chromatography. J Biochem Biophys Methods 56(1):291–296

Wacker M, Linton D, Hitchen PG, Nita-Lazar M, Haslam SM, North SJ, Panico M, Morris HR, Dell A, Wren BW, Aebi M (2002) N-linked glycosylation in *Campylobacter jejuni* and its functional transfer into *E. coli*. Science 298:1790–1793

Wacker M, Wang L, Kowarik M, Dowd M, Lipowsky G, Faridmoayer A, Shields K, Park S, Alaimo C, Kelley KA, Braun M, Quebatte J, Gambillara V, Carranza P, Steffen M, Lee JC (2014) Prevention of *Staphylococcus aureus* infections by glycoprotein vaccines synthesized in *Escherichia coli*. J Infect Dis 209(10):1551–1561. doi:10.1093/infdis/jit800

Wade TK, Saksena R, Shiloach J, kovac P, Wade WF (2006) Immunogenicity of synthetic saccharide fragments of *Vibrio cholerae* O1 (Ogawa and Inaba) bound to exotoxin A. FEMS Immunol Med Microbiol 48:237–251. doi:10.1111/j.1574-695X.2006.00143.x

Wang LX, Costantino P, Geng Y, Zhang H (2014) Anti-carbohydrate HIV vaccine design. In: Pantophlet R (ed) HIV glycans in infection and immunity. Springer, New York

Widmalm G (2013) A perspective on the primary and three-dimensional structures of carbohydrates. Carbohydr Res 378:123–132

Wieruszeski JM, Talaga P, Lippens G (2005) Development of a high-resolution magic-angle spinning nuclear magnetic resonance identity assay of the capsular polysaccharide from *Haemophilus influenzae* type b present in cetavlon precipitate. Anal Biochem 338(1):20–25

Winblad B, Andreasen N, Minthon L, Floesser A, Imbert G, Dumortier T, Maguire RP, Blennow K, Lundmark J, Staufenbiel M, Orgogozo JM, Graf A (2012) Safety, tolerability, and antibody response of active Aβ immunotherapy with CAD106 in patients with Alzheimer's disease: randomised, double-blind, placebo-controlled, first-in-human study. Lancet Neurol 11 (7):597–604. doi:10.1016/S1474-4422(12)70140-0

Wishart DS (2013) Characterization of biopharmaceuticals by NMR spectroscopy. Trends Analyt Chem 48:96–111

Wu X, Bundle DR (2005) Synthesis of glycoconjugate vaccines for *Candida albicans* using novel linker methodology. J Org Chem 70:7381–7388. doi:10.1021/jo051065t

Wu X, Lipinski T, Carrel FR, Bailey JJ, Bundle DR (2007) Synthesis and immunochemical studies on a *Candida albicans* cluster glycoconjugate vaccine. Org Biomol Chem 5(21):3477–3485. doi:10.1039/b709912f

Xie O, Pollard A, Mueller JE, Norheim G (2013) Emergence of serogroup X meningococcal disease in Africa: need for a vaccine. Vaccine 31(27):2852–2861. doi:10.1016/j.vaccine.2013.04.036

Xin H, Cartmell J, Bailey JJ, Dziadek S, Bundle DR, Cutler JE (2012) Self-adjuvanting glycopeptide conjugate vaccine against disseminated candidiasis. PLoS ONE 7:e35106. doi:10.1371/journal.pone.0035106

Xin H, Dziadek S, Bundle DR, Cutler JE (2008) Synthetic glycopeptide vaccines combining β-mannan and peptide epitopes induce protection against candidiasis. Proc Natl Acad Sci USA 105(36):13526–13531

Xu Q, Abeygunawardana C, Ng AS, Sturgess AW, Harmon BJ, Hennessey JP Jr (2005a) Characterization and quantification of C-polysaccharide in *Streptococcus pneumoniae* capsular polysaccharide preparations. Anal Biochem 336:262–272

Xu Q, Klees J, Teyral J, Capen R, Huang M, Sturgess AW, Hennessey JP, Washabaugh M, Sitrin, Abeygunawardana C (2005b) Quantitative nuclear magnetic resonance analysis and characterization of the derivatized *Haemophilus influenzae* type b polysaccharide intermediate for PedvaxHIB. Anal Biochem 337(2):235–245. doi:10.1016/j.ab.2004.11.019

Yu Ip CC, Manam V, Hepler R, Hennessey JP Jr (1992) Carbohydrate composition analysis of bacterial polysaccharides: optimized acid hydrolysis conditions for HPAEC-PAD analysis. Anal Biochem 201:343–349

Zandomeneghi G, Ilg K, Aebi M, Meier BH (2012) On-cell MAS NMR: physiological clues from living cells. J Am Chem Soc 134(42):17513–17519

Zartler ER, Porambo RJ, Anderson CL, Chen LH, Yu J, Nahm MH (2009) Structure of the capsular polysaccharide of *pneumococcal* serotype 11A reveals a novel acetylglycerol that is the structural basis for 11A subtypes. J Biol Chem 284(11):7318–7329

Zhang F, Lu YJ, Malley R (2013) Multiple antigen-presenting system (MAPS) to induce comprehensive B- and T-cell immunity. Proc Natl Acad Sci USA 110(33):13564–13569

Zon G, Szu S, Egan W, Robbins JD, Robbins JB (1982) Hydrolytic stability of pneumococcal group 6 (type 6A and 6B) capsular polysaccharides. Infect Immun 37(1):89–103

Zou W, Jennings HJ (2009) Preparation of glycoconjugate vaccines. In: Guo Z, Boons G-J (eds) Carbohydrate-based vaccines and immunotherapies. Wiley, Hoboken

Chapter 9
Vaccines in Research and Development: New Production Platforms and New Biomolecular Entities for New Needs

John P. Hennessey Jr. and Niranjan Y. Sardesai

9.1 Introduction

Currently licensed vaccines have emanated from a relatively small number of production platforms. As shown in Table 9.1, these platforms have produced a large number of licensed products and are currently being used to develop a significant number of new vaccines that are currently under clinical evaluation.

Each of the production platforms used to make currently licensed vaccines have been discussed in previous chapters to elaborate the analytical standards and challenges inherent to these platforms. As such, each of these platforms has an established precedent that guides analytical development of new products using these respective platforms. That is not to say that each new vaccine does not come with its own unique challenges, but the precedent products become both a starting template for development and an expected regulatory standard.

9.1.1 Vaccines in Clinical Development Based on New Production Platforms

Over the past 30 years, basic research efforts have defined creative new ways to engage the human immune system to combat both infectious and noninfectious diseases. This has resulted in hundreds of new vaccines undergoing preclinical development. These efforts have also spurred on parallel development of new

J.P. Hennessey Jr. (✉)
NovaDigm Therapeutics, Inc., Grand Forks, ND, USA
e-mail: john_hennessey@novadigm.net

N.Y. Sardesai
Inovio Pharmaceuticals, Blue Bell, USA

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6_9

Table 9.1 Production platforms used for antigens used in manufacture of licensed vaccines and being used to develop new vaccines that are currently under clinical evaluation

Production platform	Biomolecular entity	Examples of pathogens and biomolecular entities used as vaccine immunogens in licensed vaccine products	New vaccines in clinical development based on similar platforms (phase of clinical evaluation)
Whole virus	Live, attenuated	Measles virus	Respiratory syncytial virus (Ph 1)
		Mumps virus	
		Rubella virus	
		Varicella virus	
		Influenza virus	
		Rotavirus	
		Yellow fever virus	
	Inactivated	Polio virus	–
		Hepatitis A virus	
		Influenza virus	
		Hepatitis B virus (discontinued)	
		Rabies virus	
Subunit	Proteins	*Diphtheria* toxoid	–
		Tetanus toxoid	
		Influenza	
	Polysaccharides	*Haemophilus influenza* capsular polysaccharide (CP)	–
		Streptococcus pneumoniae CP	
		Neisseria meningitidis CP	
Recombinant proteins	Proteins	Hepatitis B virus-like particle (VLP)	*Candida albicans* (Ph 1–2)
		Human papilloma virus VLP	*Clostridium difficile* (Ph 1)
			Chlamydia trachomatis and
			Chlamydophila pneumoniae (Ph 1)
			Mycobacterium tuberculosis (Ph 2)
			Pseudomonas aeruginosa (Ph 1–2)
			Staphylococcus aureus (Ph 1–2)

(continued)

Table 9.1 (continued)

Production platform	Biomolecular entity	Examples of pathogens and biomolecular entities used as vaccine immunogens in licensed vaccine products	New vaccines in clinical development based on similar platforms (phase of clinical evaluation)
Conjugates	Polysaccharide-protein	*H .influenzae* CP-tetanus toxoid conjugate	*Staphylococcus aureus* (Ph 1–2)
		S. pneumoniae CP-diphtheria toxin mutant cross-reactive material (CRM197) conjugate	Pathogenic *Escherichia coli* (Ph 1)
		Meningitis CP-CRM197 conjugate	

technologies, new production platforms, and new biomolecular entities as vaccine active components to create vaccines to address unmet medical needs. In many cases, the resulting development candidates have new physicochemical and biological features that have not yet been considered or addressed for currently licensed vaccine products. Specific examples of these new platforms and vaccine active components produced by them are provided in Table 9.2.

In this chapter, we will highlight some of the new biomolecular entities that have advanced to clinical development and therefore require near-term thinking about evolving the analytical evaluation of these new entities to include the hallmarks of release testing, stability evaluation, and molecular characterization that are required of candidates advancing to Phase 3 clinical development and licensure.

We will not address viral-based vectors at this time as the more compelling use of this technology tends to be gene therapy applications. We will also not address

Table 9.2 New production platforms and biomolecular entities being used in new vaccines in clinical development

Production platform	Biomolecular entity	Examples of pathogens and biomolecular entities used as vaccine immunogens
Whole organism host producing parasites	Live attenuated parasite	Malaria (sporozoite)
		Leishmania (promastigotes)
Yeast expression of recombinant fungal proteins	Fungal glycoprotein	*Candida albicans*
Bacterial expression of recombinant polynucleotides	Double-stranded DNA plasmids RNA	HPV, HIV, HCV, Influenza, HBV, malaria, and various cancer vaccines
		Various cancer vaccines
Replication-incompetent virus with a complementary host cell line	Viral-based vector carrying a transgene for the target protein	HIV-AIDS

RNA-based vaccines, which have established human proof of concept in cancer immunotherapeutic applications, but have not yet established a clear target indication (Ulmer et al. 2012).

9.1.2 Vaccines in Clinical Development Based on Whole Organisms

9.1.2.1 Overview

Vaccines against parasitic diseases represent an area of enormous need, much research activity, but limited success. Parasitic disease vaccines represent, as a class, one of the biggest global unmet medical needs of any infectious disease category, impacting billions of people and resulting in millions of deaths each year (World_Health_Organization 2013a). The major disease targets that have produced clinical-stage vaccine candidates include amoebiasis, hookworm, leishmaniasis, malaria, schistosomiasis and trypanosomiasis. While all of these disease targets have seen some vaccine development efforts with recombinant protein vaccines, malaria, and leishmaniasis have seen significant development of vaccines based on the use of live attenuated or killed whole organisms, respectively. There is limited precedent with this vaccine modality in the modern era, with live attenuated or killed bacterial vaccines offering a limited precedent (see Chaps. 5 and 6, respectively). Future success with malaria or leishmania vaccines could spur on efforts to evaluate whole organism-based vaccines for other parasitic disease targets as well. As such, analytical development and quality control issues specific to this vaccine modality will be addressed below.

Malaria. Malaria is a disease caused by a protozoan parasite that is transmitted by mosquitos. *Plasmodium falciparum* is the main protozoal species of concern, but other species of *Plasmodium* can also cause disease. For 2012, it is estimated that 207 million people were afflicted with malaria and 627,000 people died of malaria, including 482,000 children under the age of five (World_Health_Organization 2013b).

Sanaria, with support of a large number of international collaborators, is developing a live attenuated malaria vaccine candidate based on irradiated *P. falciparum* sporozoites (Fig. 9.1). This vaccine is based on the use of sporozoites that are manually harvested from the salivary glands of irradiated mosquitos, isolated by a series of filtration and centrifugation steps and then stored frozen (Epstein et al. 2011a). While scale-up remains an issue with the manual harvest of salivary glands from irradiated mosquitos, this vaccine, which is administered intravenously, has been demonstrated to be safe, immunogenic and protected human vaccinees from controlled malaria infections (Epstein et al. 2011b; Seder 2013).

Leishmaniasis. Leishmaniasis is a disease caused by a flagellated protozoan that is transmitted by the phlebotomine sandfly. Many species of *Leishmania* can cause disease, but specific species appear to be endemic to certain regions and are primarily

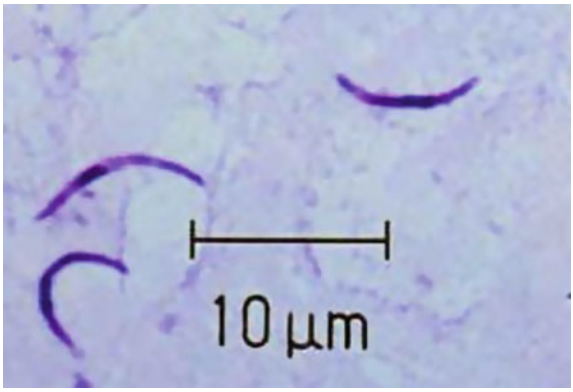


Fig. 9.1 *Plasmodium falciparum* sporozoites (picture from http://www.medicine.mcgill.ca/tropmed/old/lecture2%20malaria.htm)

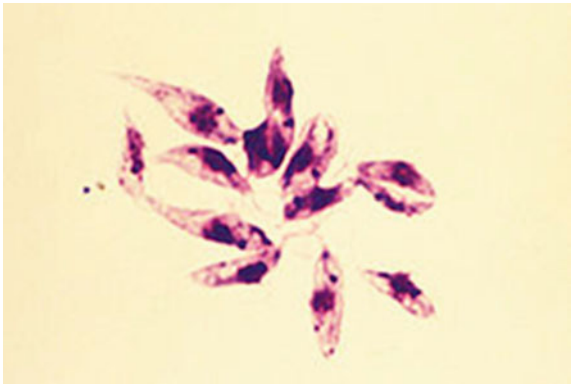


Fig. 9.2 *Leishmania tropica* promastigotes (picture from http://en.wikipedia.org/wiki/Leishmania)

responsible for disease in that region. There are currently about 12 million people afflicted with leishmaniasis and localized epidemics can have a mortality rate of up to 10 %. Leishmaniasis occurs mainly in developing nations in the tropic regions.

One of the few vaccine candidates for leishmaniasis that has been tested in humans is one based on killed (autoclaved) whole promastigotes produced in cell-free culture. Promastigotes (Fig. 9.2) are the flagellated form of the protozoan that occurs in the midgut of the sandfly. This form of vaccine has been evaluated over the past 20 years by a variety of investigators, mainly in the middle east. None of the vaccine candidates so far evaluated in humans have proven protective. However, given that recovery from natural infection provides lifelong protection from disease, a killed whole parasite vaccine seems to offer the most promise of initial success (Alimohammadian et al. 2002; Bahar et al. 1996).

The malaria and leishmaniasis whole parasite vaccines share several common features that suggest that they may utilize a similar approach for analytical development and quality control. Given that the malaria vaccine developed by Sanaria has had the most extensive evaluation from analytical, clinical, and regulatory perspectives, it will be used as an example of how to approach some of the analytical challenges inherent to this vaccine modality.

9.2 Analytical Challenges

Analytical development of a vaccine product based on a complex component such as irradiated sporozoites has to have a targeted focus. Assuming that a "batch" is defined as a final pool of harvested sporozoites, the key analytical parameters for the bulk drug substance (BDS) would focus on purity, dose, potency, and proof of attenuation or inactivation (i.e., inability to replicate) (Epstein et al. 2011a). While additional parameters can be very informative as to the nature of the vaccine and the consistency of manufacture, these four parameters are central to the quality control of the vaccine.

Purity. The purity of the BDS is largely based on demonstration of sterility (e.g., bioburden <1 colony forming unit per mL) and is dependent on growth of the host (i.e., mosquitos) and the parasite (i.e., sporozoites) under sterile conditions. This is similar to considerations required for a cell culture-based production process, requiring closed production processes as terminal sterile filtration is not possible.

Dose. The dose of the final vaccine product is based on an assessment of dose per unit volume in the BDS and appropriate dilution during preparation of the final formulated bulk vaccine. The basic unit for dosing this vaccine and the key parameter for manufacture (dilution) of the vaccine is the number of sporozoites per unit volume. This measurement is based on visual counting of sporozoites by trained technicians using a standard operating procedure, which likely includes some consideration for the integrity (wholeness) of the sporozoites to capture variations in processing that might impact the potency of the resulting vaccine.

Potency. Potency of the vaccine is assessed using a cell-based assay in which hepatocytes were incubated with sporozoites and shown to express *P. falciparum* liver stage antigen-1 (PfLSA-1), a protein that is not expressed in sporozoites, but is expressed in liver cells after infection. This assay provides an efficient assessment that the sporozoites can in fact "infect" the liver cells, much as they would in a natural infection. The infection process is fundamental to the induction of an immune response to the live attenuated sporozoite vaccine and presumably to inducing a protective response. Demonstration of the stability indicating nature of the potency assay is also an essential analytical milestone that will need to be achieved prior to advancing to Phase 3 clinical studies.

Attenuation. Additionally, adequacy of attenuation is checked on each bulk lot of sporozoites by showing that late liver and asexual erythocytic stage proteins are not expressed at 6 days postexposure of the hepatocytes to the sporozoites. This measure ensures that there is no replication of the sporozoites that could propagate disease.

Specifications. Quality control of this vaccine has no modern-day precedent, given the complexity of the active ingredient (the irradiated sporozoite) and the need for it to remain in a functional, intact state but incapable of replication. Development of specifications for release and stability testing for each of these measures in the Sanaria vaccine is likely still in a preliminary state, relying on interim specifications until such point as sufficient data is available on multiple batches of BDS and final vaccine to allow a data-driven determination of sources of

variability and suitability-for-use limits. A challenge for this type of vaccine product and in fact most vaccine products will be obtaining adequate clinical dose ranging data to support a lower limit of dose or potency that will result in efficacy to prevent disease as well as an upper limit of dose or potency above which adverse events become an issue. Without adequate clinical data to establish these limits, there is the risk that the vaccine specifications will be artificially narrow and perhaps without adequate consideration of the variability inherent to the production, isolation, stability, and analysis of the live attenuated sporozoites.

9.2.1 Vaccines in Clinical Development Targeting Fungal Pathogens

9.2.1.1 Overview

Vaccines against fungal pathogens have a long and diverse history of preclinical development, but candidates that make it into clinical studies have been few and far between (Edwards 2012). Vaccines for fungal pathogens have been getting an increasing amount of attention over the past few decades due to rising concern about the impact of fungal pathogens on human health and survival. Indeed, invasive fungal diseases can have a mortality rate of up to 50 % despite treatment with the licensed array of antifungal drugs (Cleveland et al. 2012) and more people in the US are killed by invasive candidiasis each year than are killed by methicillin-resistant *Staphylococcus aureus* (MRSA) (Cleveland et al. 2012; Wisplinghoff et al. 2004). Except for a few studies done in the 1980s, it has only been in the past few years that fungal vaccine candidates have reached the preclinical milestones that permit clinical evaluation. This is despite the fact that serious fungal diseases are on the rise as is resistance to antifungal drugs.

Candida. *Candida* is the only fungal pathogen for which clinical development of a vaccine is evident. *C. albicans* accounts for over half of all candidal disease and is the third most common pathogen in hospital ICUs and the fourth most common hospital-associated pathogen (Wisplinghoff et al. 2004). At present, NovaDigm Therapeutics has initiated a Phase 2a study of a vaccine based on recombinant agglutinin-like sequence 3 (rAls3) protein produced by *Saccharomyces cerevisiae*, and Pevion has completed a Phase 1 study of a vaccine based on recombinant secreted aspartyl proteinase 2 (rSap2) produced by *Escherichia coli*.

Analytical development of products based on recombinant proteins made in bacterial and yeast host cell lines are based on well-established precedent. However, rAls3 represents the first instance of a recombinant fungal protein made in a yeast cell line, thus allowing fungal posttranslational processing to generate a new breed of recombinant glycoprotein. The rAls3 antigen incorporates a large number of short-chain O-linked glycans that pose a new challenge for analytical development that combines the concerns of a recombinant protein (Chap. 3) with some of the challenges seen in polysaccharide-protein conjugate vaccines (see Chap. 9).

9.3 Analytical Challenges for rAls3

rAls3 has been shown to contain ~20 % carbohydrate by weight, with all of it consisting of O-linked oligomannose (Hennessey and Shabb in progress). Given that 27 % of the amino acids in this protein are serine or threonine, rAls3 presents a puzzle for analytical definition as well as for evaluation of structure-function analysis. The key analytical parameters for the BDS would focus mainly on carbohydrate analysis and on intact mass and the molecular variants observed. These parameters will provide critical elements for molecular definition of this vaccine antigen and future measurements on which to base evaluation of lot-to-lot consistency and stability.

Carbohydrate analyses. Analysis of the carbohydrate content of BDS lots is well within the realm of standard methodologies used with other glycoproteins. Acid hydrolysis of the glycoprotein yields monosaccharides that are amenable to HPLC analysis, showing that only mannose can be detected and that it accounts for ~15 % of the mass of the glycoprotein (Hennessey and Shabb in progress). This analysis is as important for what it does not show as for what it does in that it doesn't show the presence of residual glucose, β-glucans, or N-linked oligosaccharides, all of which can be found in fungal/yeast protein preparations.

In contrast, evaluation of the oligomeric structure of the carbohydrate entities cannot rely on the convenient enzymatic treatments used to liberate oligosaccharides from N-linked attachment sites. Instead, a mild β-elimination followed by Michael addition with dithiothreitol (BEMAD) (Wells et al. 2002) can be used to release the oligosaccharides from the O-linked attachment sites for subsequent HPLC analysis. Such an analysis applied to rAls3 shows that ~85 % of the released saccharides are either monomers or dimers of mannose, with the remainder composed of mannose trimers, tetramers, and pentamers (Hennessey and Shabb in progress).

While both of these analyses are important to defining key features of the rAls3 glycoprotein and its consistency of manufacture, neither is likely to be discriminating enough to be used as a quality control measure or as a measure of stability, given the overall complexity of the glycoprotein preparation.

Intact mass. The intact mass profile of rAls3 presents as a broad array of glycoforms by mass spectrometry analysis (Fig. 9.3) that is based on a complex distribution of O-linked mannose monomers and oligomers distributed over a large number of potential glycosylation sites (Hennessey and Shabb in progress). The intact mass profile supports that the rAls3 BDS contains ~30 significant glycoforms based simply on the number of hexose units per protein molecule. Considering the number of glycosylation site variants, this protein presents a significant challenge to evaluation of consistency of manufacture. Key to addressing this challenge, measures to reflect the carbohydrate portion of the molecule and the molecular variants of the glycoprotein are needed and will be central to the quality control of the vaccine.

Molecular variants. The mass spectrometry profile (Fig. 9.3) gives a fairly sensitive fingerprint for the intact mass variants contained in a BDS preparation, showing significant amounts of glycoproteins containing 60–90 hexose units per protein molecule. This profile should also be sensitive to any N- or C-terminal

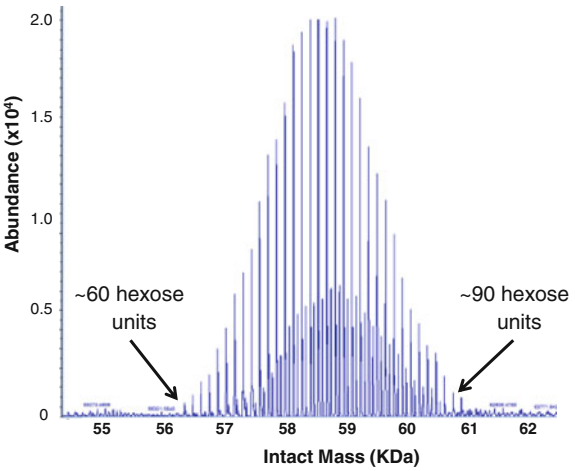


Fig. 9.3 Mass spectrometry profile of intact rAls3p-N shows a broad distribution of glycoforms (NovaDigm Therapeutics, unpublished data)

proteolytic events in that an intact mass profile similar to the original would be then be seen at lower molecular masses. Both of these attributes of this analysis suggest that this assay would be a suitable quality control assay for BDS release as well as be a stability-indicating assay if glycolytic or proteolytic influences were encountered.

Evaluation of glycosylation site variants for this glycoprotein makes for a very interesting characterization study, but the complexity of the number of saccharide oligomer species and the large number of potential glycosylation sites precludes this from being a practical undertaking for application to multiple lots. As such, a high-resolution measure of molecular variants may be all that is needed to provide reasonable proof of consistent manufacture of the glycoprotein.

Finally, determination of the functional relevance of this complex posttranslational modification will be difficult, particularly since the immunological response to this glycoprotein includes both B and T cell responses. As such, assessments of dose and potency will initially be measured by protein content and immune response in a mouse model, which is a common starting point for many vaccines, but will need to evolve to a more refined analysis as additional data, both in vitro, preclinical and clinical, is gathered and evaluated.

9.3.1 Vaccines in Clinical Development Based on Double-Stranded DNA Plasmids

9.3.1.1 Overview

DNA immunization as a concept was first pioneered in the early 1990s (see Montgomery et al. 1993; Ulmer et al. 1994) and, owing to its simplicity, gained widespread recognition. Indeed, the idea that the basic unit of molecular biology,

i.e., a DNA plasmid, can be engineered to deliver different antigenic payloads through synthetic recombinant DNA approaches, and furthermore be combined with the power of genomics to design and optimize potent immunogens, providing a powerful vaccine development approach to researchers. Over the years, researchers developed methods to improve the potency of the DNA constructs through many approaches, including gene optimization, RNA optimization, use of leader sequences, use of DNA-encoded cytokines and co-stimulatory molecules, novel formulations, and improved in vivo gene delivery methods—both physical (gene gun, electroporation, sonoporation) and chemical (liposomes, transfection agents) (Kutzler and Weiner 2008; Sardesai and Weiner 2011). DNA plasmid manufacturing typically relies on bacterial hosts for production and BDS lots can be easily characterized to the level of molecular identity through genetic sequencing.

There are four DNA vaccines/therapy products licensed for use in animals worldwide (Kutzler and Weiner 2008) and the most advanced human vaccine products are currently in Phase 2–3 clinical development, with over 800 clinical trials conducted worldwide covering over 20,000 subjects across infectious disease and cancer targets (www.clinicaltrials.gov; keyword search "DNA Vaccine"). As such, while the regulatory experience for early stage clinical development is vast, the lack of product approved for human use means that the regulatory pathways for licensure of DNA-based products still need to be tested. DNA vaccine development involves several analytical measures that are unique to the realm of vaccines and as such will be a critical part of those technical and regulatory discussions.

A complete listing of disease targets for which the DNA vaccines are being developed is beyond the scope of this chapter, but we report two examples, human papillomavirus (HPV) and human immunodeficiency virus (HIV), to illustrate the versatility of the DNA technology.

HPV. Over 100 different types of human papillomavirus strains have been described and 15 types have been positively identified as having carcinogenic potential. Two prophylactic vaccines (Gardasil® and Cervarix®) have been licensed that target the major oncogenic serotypes 16 and 18 (Gardasil® additionally targets serotypes 6 and 11) based on the induction of antibody responses to the respective viral L1 proteins configured in a virus-like particle (VLP). These VLP-based vaccines are described in Chap. 3 in this book. However, the L1-based preventive vaccines are ineffective once the person is infected with HPV and the virus transforms the basal cervical cells into dysplastic or oncogenic cells through the integration into the host genome. This oncogenic transformation is driven through two small proteins E6 and E7 (approximately 110 aa each); and in turn, the development of vaccines against E6 and E7 as a means to treat HPV infection and/or cancer has been an active area of research. Several groups have used proteins, peptides, viral vectors, *Listeria* vector, and DNA-based approaches to develop E6 and E7 targeted vaccines (Morrow et al. 2013). Data from a two DNA plasmid-based approach encoding four antigens (E6 and E7 antigens from both HPV 16 and 18) was recently reported by Inovio Pharmaceuticals. The researchers showed that the vaccine when delivered with in vivo electroporation in women with a previous history of cervical lesions, was safe, well tolerated, and elicited high levels of

antigen-specific antibody and CD8+ T cell responses. The CD8+ T cells were also functional in their ability to kill infected cells in the majority of vaccinees, suggesting proof of concept to develop a viable therapeutic vaccine (Bagarazzi 2012).

HIV. HIV represents one of the top three global health threats and presents a unique set of challenges to effective vaccine development both because of its pathobiology, its ability to subvert the host immune systems, as well as its ability to mutate rapidly and escape long-term control. Many groups have focused on using DNA plasmids, viral-based vectors (VV), protein, and prime-boost approaches (DNA-VV; DNA-protein; VV-protein, etc.) in the clinic as a way to target the induction of cellular and/or humoral immune responses that are expected to be important to dealing with HIV infections.

While DNA plasmid vaccines are being evaluated for several other disease indications (see Table 9.2), these additional indications do not bring about additional analytical issues related to the vaccine beyond the two examples given above.

9.4 Analytical Challenges

DNA plasmid vaccines are a new vaccine modality that presents new analytical issues to be addressed for the characterization and quality control of a licensed vaccine product. The fact that the basic components of the active entity (nucleotides) differ from the more familiar amino acids found in protein entities requires a ground-up evaluation of this new product modality.

Plasmid DNA consists of two complementary strands of polynucleotides that are uniquely defined by their primary structure (nucleotide sequence) and form supercoiled macromolecular structures. This is analogous to the compositional and structural characteristics of proteins. Posttranslational modification of DNA can also occur and many of the quality control concerns that are typical of a protein BDS are also of concern for a DNA plasmid BDS. The FDA and the WHO have each issued guidance-for-industry documents outlining specific considerations for the production and control of DNA plasmid vaccines (US_Food_&_Drug_Administration 2007; World_Health_Organization 2007).

The key analytical parameters of relevance to DNA plasmid vaccines are similar in concept to those used for other modalities, but require analytical technologies appropriate to polynucleotides and are mainly focused on purity and integrity of the BDS (purified plasmid) and potency of the vaccine product.

Plasmid purity. For plasmid DNA products the presence of host cell proteins and other host nucleic acids, i.e., genomic DNA, and RNA (mRNA, tRNA, rRNA) needs to be evaluated. Although the sizes and physicochemical properties of the host cell residuals are sufficiently different to allow for separation during purification, their characterization and quantification are nevertheless important to assure the sufficiency and consistency of the purification process. Preliminary limits for each of these components are often established at <1 % early in development per

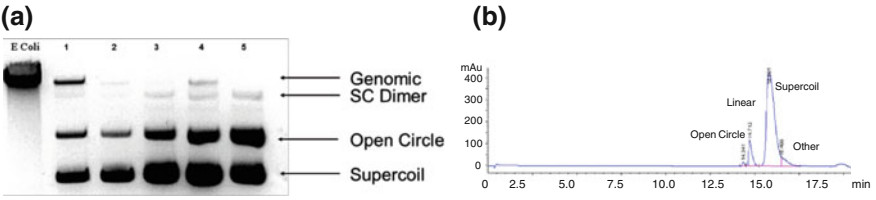


Fig. 9.4 Plasmid integrity is evaluated by agarose gel electrophoresis (*panel A*) or by HPLC analysis (*panel B*) (courtesy of D. Peterson, VGXI, Inc.)

the FDA guidance. Endotoxin is an important contaminant of bacterial fermentation processes and sensitive assays to detect residual endotoxin must be included in the analytical characterization package. The FDA guidance indicates a target of less than 40 EU/mg as detected via the Limulus Amebocyte Lysate (LAL) test.

Plasmid integrity. Double-stranded DNA produced by microbial fermentation is in the form of closed-circular double-stranded supercoiled DNA through the intermediacy of host enzymes. A portion of this may be nicked (cut in one of the strands; to produce a closed-circular relaxed form of the same DNA); or linearized (cuts in both strands in close proximity). Sometimes, a plasmid dimer may be produced due to concatenation of plasmids. Figure 9.4 demonstrates the relative mobility of the different forms of plasmid DNA on an agarose gel (Panel A) and the retention times of the different species by reverse-phase HPLC (Panel B). Each of these forms theoretically may have different transfection efficiencies for insertion into host cells leading to differences in immunogenicity and/or plasmid stability, and may be used as one of the measures of plasmid purity. Thus, it is recommended that a bulk release criteria be established to include a minimum % supercoiled specification (typically >80 %).

Potency Assay. The FDA guidance document provides developers considerable flexibility in the choice of assays early in development. Assays for potency for DNA vaccines may include in vitro measures of transfection of the DNA plasmid into host cells or the transcription and/or translation of the plasmid products into the relevant encoded protein antigens. They may also include in vivo assays to measure immunogenicity of the transgene protein encoded by the DNA plasmid. Recently, Mahajan et al. (2008) reported a Taqman RT-PCR-based in vitro potency assay for DNA plasmid products and Badger et al. (2011) reported the development of a flow cytometry based potency assay for a Venezuelan equine encephalitis virus DNA plasmid vaccine that assesses the in vivo expression of the transgene product in host cells. Each approach has its benefits in being able to serve as a viable potency assay. Vaccine developers need to consider the analytical robustness of assays used to measure activity at the different levels in this continuum while developing a viable potency assay, i.e., whether to measure potency at the DNA level, mRNA transcript level (transcription), protein expression level (translation), antigen processing and presentation level (MHC class I/II), induction of immune responses level (antigen-specific T cells and/or antibodies), or ultimately at the level of biological efficacy in an animal model. Ultimately, as with other vaccine approaches the choice of assay

for measuring potency of DNA plasmid vaccines will also benefit from and be informed by the identification of a clear correlate of protection for each disease indication.

In general, DNA plasmid-based products have established a strong foundation of analytical development that establishes a well-founded understanding of the composition, structure and functional activity of these vaccine active components, which in turn will allow suitable quality control of these products as they enter the market of highly regulated commercial vaccines. That said, it is only a matter of time before the first DNA plasmid-based products are licensed for human use and there is a better defined or precedent setting analytical/regulatory path for the development of future DNA plasmid-based products.

9.5 Future Vaccines

We have identified several new manufacturing platforms that are currently bringing new product modalities into play to explore new vaccines for prophylactic and therapeutic applications against infectious diseases as well as other causes of disease. Each of these new platforms brings with it analytical considerations that are familiar and with extensive precedent for addressing them. However, they also bring forward new analytical issues that are critical to the definition, understanding and quality control of these new entities. Undoubtedly, additional new platforms will come into play as vaccine antigens. For these too, we will need to be vigilant, open-minded, and creative to identify these issues and find creative ways to monitor them.

References

Alimohammadian MH et al (2002) The role of BCG in human immune responses induced by multiple injections of autoclaved Leishmania major as a candidate vaccine against leishmaniasis. Vaccine 21(3–4):174–180

Badger CV et al (2011) Development and application of a flow cytometric potency assay for DNA vaccines. Vaccine 29(39):6728–6735

Bagarazzi ML et al (2012) Immunotherapy against HPV16/18 generates potent TH1 and cytotoxic cellular immune responses. Sci Transl Med 4(155):155ra138

Bahar K et al (1996) Comparative safety and immunogenicity trial of two killed Leishmania major vaccines with or without BCG in human volunteers. Clin Dermatol 14(5):489–495

Cleveland AA et al (2012) Changes in incidence and antifungal drug resistance in candidemia: results from population-based laboratory surveillance in Atlanta and Baltimore, 2008–2011. Clin Infect Dis 55(10):1352–1361

Edwards JE Jr (2012) Fungal cell wall vaccines: an update. J Med Microbiol 61(Pt 7):895–903

Epstein JE et al (2011a) Live attenuated malaria vaccine designed to protect through hepatic CD8 (+) T cell immunity. Science 334(6055):475–480

Epstein JE et al (2011b) Live attenuated malaria vaccine designed to protect through hepatic CD8 (+) T cell immunity. Science. www.sciencemag.org/cgi/content/full/science.1211548/DC1

Hennessey JP Jr, Shabb J (in progress) Structure-function analysis of the recombinant Als3 glycoprotein used as a vaccine antigen

Kutzler MA, Weiner DB (2008) DNA vaccines: ready for prime time? Nat Rev Genet 9 (10):776–788

Mahajan R et al (2008) A TaqMan reverse transcription polymerase chain reaction (RT-PCR) in vitro potency assay for plasmid-based vaccine products. Mol Biotechnol 40(1):47–57

Montgomery DL et al (1993) Heterologous and homologous protection against influenza A by DNA vaccination: optimization of DNA vectors. DNA Cell Biol 12(9):777–783

Morrow MP, Yan J, Sardesai NY (2013) Human papillomavirus therapeutic vaccines: targeting viral antigens as immunotherapy for precancerous disease and cancer. Expert Rev Vaccines 12 (3):271–283

Sardesai NY, Weiner DB (2011) Electroporation delivery of DNA vaccines: prospects for success. Curr Opin Immunol 23(3):421–429

Seder RA et al (2013) Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. Science 341(6152):1359–1365

Ulmer JB et al (1994) Protective immunity by intramuscular injection of low doses of Influenza virus DNA vaccines. Vaccine 12(16):1541–1544

Ulmer JB et al (2012) RNA-based vaccines. Vaccine 30(30):4414–4418

US_Food_&_Drug_Administration (2007) Guidance for industry: considerations for plasmid DNA vaccines for infectious disease indications. http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm091968.pdf

Wells L et al (2002) Mapping sites of O-GlcNAc modification using affinity tags for serine and threonine post-translational modifications. Mol Cell Proteomics 1(10):791–804

Wisplinghoff H et al (2004) Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis 39(3):309–317

World_Health_Organization (2007) Guidelines for assuring the quality and non-clinical safety evaluation of DNA vaccines: annex 1. http://www.who.int/biologicals/publications/trs/areas/vaccines/dna/Annex%201_DNA%20vaccines.pdf?ua=1

World_Health_Organization (2013a) Initiative for vaccine research: parasitic diseases. http://www.who.int/vaccine_research/diseases/soa_parasitic/en/index.html

World_Health_Organization (2013b) World malaria report. http://www.who.int/malaria/media/world_malaria_report_2013/en/

Chapter 10
Role of Analytics in Viral Safety

Rebecca L. Sheets and Paul A. Duncan

10.1 Introduction

Virtually all vaccines are prepared on a biological substrate and/or in a biological growth medium. The conditions used to prepare the vaccine are not only ideal for growth of the vaccine organism, but also capable of propagating adventitious (inadvertent) microbiological contaminants. Such contaminants may include bacteria, fungi, mycobacteria, mollicutes (mycoplasmas, spiroplasmas, acholeplasmas), agents of transmissible spongiform encephalopathies (e.g., bovine spongiform encephalopathy (BSE)), and viruses, including bacteriophage. Of particular difficulty for current detection methods is addressing the vast array of viral organisms, which vary considerably in terms of size, shape, content of lipid membranes (enveloped or nonenveloped), and content of nucleic acid (DNA, RNA, single-stranded, double-stranded, contiguous, or segmented genome); as well as varying in their sensitivity to inactivation procedures or efficiency of removal (by purification) procedures.

Testing methods for bacteria, fungi, mycobacteria, and mollicutes are fairly standardized. Harmonization or convergence of test methods is increasingly occurring across regulatory regions and pharmacopeia and will not be discussed further in this chapter, except in that some of the newer methods that will be

R.L. Sheets (✉)
Division of Acquired Immunodeficiency Syndrome (AIDS), US National Institutes of Health, National Institute of Allergy and Infectious Diseases, Vaccine Research Program, Vaccine Translational Research Branch, Bethesda, MD 20892, USA
e-mail: rsheets@grimalkinpartners.com

P.A. Duncan
Merck Research Laboratories, West Point, PA 19486, USA

Present Address:
R.L. Sheets
Grimalkin Partners, Silver Spring, MD 20906, USA

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6_10

discussed may be applied to their detection, as well as to the detection of viruses. It should be acknowledged that in regards to testing for mollicutes, newer methods (polymerase chain reaction-based, with or without biological amplification) have been developed and work remains in regards to harmonization of test methods acceptable in various regulatory regions.

Unfortunately, at present there is no standardized and validated test method to detect the agents of transmissible spongiform encephalopathies, like BSE, in biological products or the raw materials used in their preparation (e.g., bovine serum). Thus, strategies to control risk of product contamination entail implementing a number of product design elements, rather than testing for presence or absence. One strategy is to eliminate, to the extent possible, exposure to animal- and human-derived raw materials. This is not always feasible, and often cannot be implemented in regards to legacy products, without risk of altering in unknown ways a product of established safety and efficacy. Foremost among the possible strategies is controlling what materials to which the product is exposed by appropriate donor screening and geographic sourcing, as well as traceability and documentation, and in the case of BSE risk, eliminating high-risk specified risk materials from the collection process at the abattoir. Information on this topic may be obtained from http://www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/ucm111476.htm and http://www.emea.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003700.pdf (EMA 2011). Taking another strategy, work by staff of the U.S. Food and Drug Administration (FDA) (Piccardo et al. 2011) has shown that many vaccine cell substrates did not propagate highly infectious BSE or variant Creutzfeld-Jacob disease agents.

The risk bacteriophage may pose to product recipients, if any, has been considered historically (in the 1970s and 1980s). While their presence may interfere with production of bacterial products, or products prepared from bacteria, their risk to humans has largely been dismissed. As such, their inadvertent presence, though not desirable, was codified as acceptable (21 CFR 630.18(a) and 630.60(c), last promulgated in 1996). Generally, the presence of bacteriophage is taken as a biomarker reflecting a previous level of bioburden in a material, which would have either been controlled (for nonsterile, nonparenteral products) or absent due to sterilization (for most raw materials and for sterile and/or parenteral products). As a consequence, testing is not generally performed to detect bacteriophage in vaccines derived from mammalian or avian cell culture.

Thus, the remainder of this chapter will focus on animal-viral testing, and the principles behind assessing viral risk and guiding testing strategies. Traditional or conventional methods, though not entirely harmonized across regulatory regions or among pharmacopeia, will be discussed in regards to the principles whereby detection is achieved. Further, newer methods, as yet not validated, standardized, or widely accepted by regulators, will also be discussed as it is anticipated that their utility will begin to be seen in short order.

Historically, bacterial vaccines were (and still are) generated by fermenting the bacterial vaccine organism and likewise, viral vaccines were and are generated by propagating the viral vaccine organism on a cell substrate, tissue taken from an

animal, or in whole organisms (e.g., embryonated hens' eggs). However, increasingly, viral vaccines may be generated from *E. coli* or from yeast; while bacterial and parasitic vaccines are being prepared in viral vectors grown on cell substrates. Thus, the lines between a "bacterial" product and a "viral" product have blurred. With this, so too have the types of microbial contaminants that may be of concern for a given product type. As a consequence, the focus of this chapter on biosafety from the perspective of animal viruses should not be taken to mean that it is solely applicable to viral vaccines.

10.2 Principles of Detection Methods

Tests for viruses currently in use include those used for clinical diagnostics in practice in the mid-twentieth century (in vivo and in vitro in tissue culture), as well as techniques derived in the latter part of the twentieth century [transmission electron microscopy (TEM), polymerase chain reaction (PCR), reverse transcriptase assays]. The older methods are based on observations of responses to viral infection made in vivo or ex vivo, whereas the newer methods are based on some physicochemical aspects of viruses. These principles will be elaborated below.

10.2.1 Detection by Physicochemical Properties of Viruses

Viruses display a wide variety of physicochemical properties. Viruses come in a variety of sizes (from $\sim$20 to 100s of nm) and shapes (icosahedral, spherical, bullet-shaped, filamentous, oblong; as well as regular in shape or irregular in shape). Some contain long spikes protruding from the capsid. Some viruses contain lipid membranes (enveloped) and some do not. Further, their nucleic acid content varies in every imaginable form. They may have dsDNA, ssDNA, dsRNA, ssRNA of the "positive" (in the same sense as mRNA) or "negative" (complementary to and requiring transcription to the sense of mRNA) sense, and genomes that encompass both RNA and DNA. Some viruses have genomes that are contiguous and some are segmented. Retroviruses have two copies of the same genome packaged into a single capsid, thus are effectively diploid, while most viruses are "haploid." This wide variety makes detection of viruses complicated, and no one traditional method readily detects all types.

10.2.1.1 Detection of Viral Structures

Although viruses come in a variety of sizes and shapes, their structures can be used to identify them. While it takes experience to recognize the difference between normal cellular structures and those of some viruses, experienced transmission electron

microscopists can distinguish between them and thus, identify the presence of viruses. There are limitations to TEM however. It is neither a sensitive nor a specific method. While the latter is to benefit in that a variety of micro-organisms, including viruses, may be detected by this method, nonspecific structures may be mistaken for viruses and viruses can be missed. Furthermore, the sensitivity in terms of limit of detection for this method requires a concentration of virus on the order of 10^5 or 10^6 particles/mL. Nonetheless, viral structures can be diagnostic if seen on TEM. So, the look of a virus' structure by TEM is one means that viruses may be detected. TEM applications for virus detection were reviewed by Roingeard (2008).

10.2.1.2 Detection of Viral Proteins

Viral proteins have some unique characteristics that permit their recognition in detection assays. One such feature is the ability of certain viral proteins to bind to and agglutinate red blood cells. Many viruses (e.g., orthomyxoviruses and paramyxoviruses) have a viral protein termed hemagglutinin, with exactly this property. Other features entail the unique biological activity of a viral protein, e.g., the ability of the polymerase of retroviruses to reverse transcribe RNA into DNA. Finally, the ability of antibodies to recognize epitopes on viral proteins may be used in immunofluorescent assays (IFA).

Hemagglutination and Hemadsorption

Hemagglutination of red blood cells (RBCs) by infectious organisms was a characteristic discovered as early as the nineteenth century. Thus, as viruses began to be manipulated and studied in the twentieth century, this characteristic was used as a means of assessing for viral infection. The ability of some viruses to adsorb to RBCs, causing clumping or agglutination, was noted in early explorations of viruses. This ability was exploited to develop an early clinical diagnostic test for viruses. These tests, either the hemagglutination of RBCs by supernatants, sera, or plasma containing viruses or the ability of infected cells in culture to hemadsorb RBCs (Shelokov et al. 1958), permit a visualization of viruses by cross-linking viruses and RBCs together in a large enough clump to be observed by the naked eye (hemagglutination) or by light microscopy (hemadsorption).

However, not all viruses contain a protein that can hemagglutinate RBCs. As a consequence, this diagnostic parameter is only useful to detect some viruses. Nonetheless, it is a broad general screen requiring little knowledge of the type of virus for which one is looking. Like TEM, it is not specific (bacteria can also hemagglutinate) and in order to visualize the process, a sufficient quantity of virus, viral proteins, or viral particles on the surface of an infected cell must be present to bring together an adequate number of RBCs to clump or hemadsorb and be recognized above background levels. Another caveat is that different viruses will hemagglutinate certain species' RBCs but not others, so if the wrong species' RBCs are

used, the hemagglutinating effect may be missed. An early study characterized the $\log_{10}$ ratio of infectious influenza virus per hemagglutinating dose to be ~4–6 (in other words, 10^4–10^6 ID50 per hemagglutinating dose; Donald and Isaacs 1954).

Viral Enzymes (Reverse Transcriptase)

Retroviruses contain a RNA genome, and have an obligatory step in the replicative life cycle in which their viral RNA genome is reverse transcribed into cDNA that integrates into a host cell as a provirus. It is from this provirus that viral mRNA transcripts are generated. Thus, retroviruses must package within their viral capsid a sufficient number of molecules of reverse transcriptase to ensure that this step of the life cycle is completed. Assays are available for detecting this enzymatic activity by monitoring for cDNA molecules thus reverse transcribed. The conventional or traditional method entails detection of incorporation of radioactively labeled deoxyribonucleotides into the nascent reverse transcribed DNA strand, although newer versions of this traditional method utilize nonradioactive labels.

A newer method was developed in the 1990s and referred to frequently as PERT or product-enhanced RT assay, a term coined by the authors of an early publication on the method (Boni et al. 1996). This method entails the use of PCR to amplify the signal of the reverse transcribed DNA. In the absence of RT enzyme, the RNA template will not be reverse transcribed to DNA and thus, no PCR amplification will occur. But, in its presence, amplicons are generated. This enhances the sensitivity of the method to detect RT by about six orders of magnitude over the conventional method. However, specificity is lost in that host DNA polymerases and authentic reverse transcriptases from host retroelements, present in all eukaryotic species, can also result in a signal in the PERT assay. Means to reduce this background in the assay have been implemented, but it remains a potential area of concern that challenges the conduct and the interpretation of the assay. Also, like all PCRs, test articles may contain nonspecific inhibitors that reduce the sensitivity of the method because they require dilution of the test article to permit the reaction to occur without inhibition.

Nonetheless, detecting this enzymatic activity of a viral RNA-directed DNA polymerase, distinguishing it from DNA-directed DNA polymerases, is a property that is used to detect retroviruses from all species.

Potentially, other enzymatic activities specific to viruses might be exploited for viral detection, but at present, such assays are neither in routine use nor in advanced development for that purpose, to our knowledge.

Binding of Antibodies

Intact virions present epitopes that can be recognized by binding of antibodies from specific hyperimmune sera or monoclonals. Infected cells may also present viral proteins on their surface permitting antibody binding. These antibodies can be

fluorescently labeled in order to perform an IFA. Alternatively, supernatant fluids, which may be contaminated with virus, can be assessed by application to an enzyme-linked immunosorbent assay (ELISA), although we are unaware that this is commonly used in direct adventitious agent testing of biologicals, even though it is commonly used in research settings and in diagnostics.

10.2.1.3 Viral Nucleic Acids

Before the advent of PCR, viral nucleic acids were detected by hybridization methods, such as Southern or Northern blotting or slot/dot blots. However, most currently used routine tests for viral nucleic acids are based on PCR. Frequently, these newer methods are referred to as nucleic acid tests (NAT). These may entail visualization of PCR amplicons by gel or Southern blotting or may entail use of more quantitative methods such as real-time PCR or Q-PCR.

For DNA viruses, direct PCR with either specific primers, conserved primers, or degenerate primers may be performed. Such tests are usually performed on extracted nucleic acids from cells that may potentially be infected, although they may also be performed on nucleic acids extracted from culture fluids or other aqueous solutions. Also, mRNAs of viruses can be detected, to suggest potential replication of a virus by expression of viral transcripts, though this is not commonly used as an adventitious agent test method. For RNA viruses, RT-PCR, wherein the initial step entails reverse transcription followed by PCR amplification from the generated DNA template, may be performed.

All of these methods rely on a high degree of sequence similarity between the probes, primers, and the viral species targeted for detection. This requires considerable knowledge about what viruses for which one should be testing, as well as sequence similarity or divergence among known viruses. Degenerate primers can be used to minimize the knowledge needed to detect a signal, but these are not currently in widespread use in the testing field, in part because of the enhanced specificity of specific primers.

10.2.2 Response of Cells or Organisms to Virus

Some viruses cause apparent infection in cells in culture and can be detected in vitro by these means. Such viruses are noted by the cytopathic effects (CPE) they cause on a culture monolayer.

Some viruses do not cause apparent infection in culture. These so-called "inapparent viruses" may be detectable by the responses of living organisms to infection. They may result in death of animals or eggs, or morbidity, which is detectable by notable signs that may be monitored.

Similarly, animals may respond to infection by mounting detectable antibody responses and this capability is exploited in the so-called mouse, rat, or hamster

antibody production assays (MAP, RAP, HAP). The antibodies generated in the relevant species are detected by IFA or ELISA methods.

10.2.2.1 Tests in Cell Cultures

The ability to successfully propagate cells in culture from explanted tissues revolutionized the ability of viruses to be propagated and researched in the twentieth century. This platform has proven to be useful for viral detection as well.

As discussed above in Sect. 10.2.1.2 and subsections, some readouts for detecting viral infection in cell culture include IFA and hemagglutination/hemadsorption. In addition, other read-outs are discussed below.

Cytopathic Effects

Once it was discovered that viruses could be propagated in explants of tissues maintained in vitro, observations were made of the CPE the viral infections caused. CPE occurs as a result of the killing of cells in a zone where viruses may have propagated from cell to cell (plaques) or the fusion (syncytia formation) of the plasma membranes of multiple infected cells. These plaques or zones of dead cells or areas of overly large cell syncytia are readily noted by light microscopy, or even by the naked eye. The spread of any viral infection across the cell sheet may depend on the nature of the virus and the infected cell's response (for instance, highly cell-associated versus secreted or released by cell lysis). Semisolid overlays, commonly used to restrict virus diffusion in some plaque assays, are not typically used in adventitious agent tests, because maximal opportunity for virus propagation aids detection.

Transformation

Transformation of cells in culture is associated with changes in cell morphology, loss of contact inhibition (the process whereby cells stop propagating when they contact too many other cells in their vicinity), and sometimes ability to grow in suspension rather than as monolayers attached to a substrate.

Although not routinely used as a viral detection test for vaccines, some viruses have the ability to transform cells in vitro. This ability derives largely from binding of viral proteins to host proteins that control cell cycling, such as p53 or RB, thus disrupting their functioning and causing the cell to lose control and grow indefinitely. Alternatively, this ability derives from being able to complement missing function in specific cell lines prepared with defective sarcomavirus (S+ L−). The result of viral transformation in culture is often seen as a focus of cells piling up without the usual contact inhibition that preserves a uniform monolayer.

Some of these viruses are also oncogenic in vivo. While an in vitro transformation assay has been used as a surrogate marker for tumorigenicity of intact cells, this is not commonly used as an adventitious agent test. However, in vitro transformation and in vivo tumorigenicity do not always correlate, so this in vitro surrogate has largely been abandoned in the field of testing of biologicals as unreliable for that purpose. Nonetheless, the ability of a virus to transform cells in culture, particularly human cells, would be concerning to regulators and may be a biological parameter warranting further research should the phenomenon be observed in the production cell line or an indicator cell line used in adventitious virus testing.

10.2.2.2 Tests in Animals

Before cell culture, viruses were propagated from animal to animal in order to study them in the experimental setting. Clinical observations of the effects viruses have on experimental animals formed the basis for early clinical diagnostics for viruses. These tests remain in use for adventitious agent testing because of historical utility, but public reports from modern testing service providers have called into question their actual utility in the era of current Good Manufacturing Practices and newer testing methodologies. Recent work has suggested that their sensitivity for detection of viruses may not be as good as previously believed (Gombold et al. 2014), despite having been relied upon for years. The benefit of such tests is that they require no prior knowledge about what virus or organism may be present in a test article nor do they require the ability of the virus to adapt to or be able to grow in cell culture.

The MAP, RAP, and HAP assays, described in Sect. 10.2.2 above, may be replaced by specific PCR tests and these replacement tests have begun to be accepted by regulators on the basis of demonstration of comparable sensitivity of detection.

Pathological Readouts

Generally, the inapparent viruses tests are performed in adult (postweaning, generally 3–4 weeks of age or older, but of a certain weight restriction that ensures they are young animals) and suckling (newborn) mice and in embryonated chicken eggs. In addition, the European Pharmacopeia requires testing viral vaccine seed lots in guinea pigs. This test is recommended in other regulatory regions in certain cases (to detect *Mycobacterium* sp., lymphocytic choriomeningitis, or Marburg virus). Finally, in certain specific cases (to detect simian Herpes B virus), rabbits might also be recommended.

An obvious sign of infection in animals or hens' eggs is death. In fact, this is the most obvious readout of the in vivo tests that are routinely employed, which require that 80 % or more of inoculated animals or eggs survive the test.

However, other pathological signs or findings may also indicate a viral infection. While animals cannot be queried like humans can about symptoms, they can be

observed for various signs. Weight loss is frequently observed in infections from which the animals recover and survive. Signs of illness may include ruffled fur or hunched posture, due to lack of normal grooming behavior or normal mobility from the animal feeling unwell. Other behavioral signs may suggest neurological impairment, particularly a sign like hind limb paralysis and its resultant impact on mobility of the animal. In the context of hens' eggs, behavioral signs are unobservable, but pocking of the chorioallantoic membrane can be indicative of viral infection. The ability of the allantoic fluids to hemagglutinate is also indicative of viral infection.

Although not frequently used in adventitious agent testing, fever can be another clinical sign suggestive of infection. This sign is used in the rabbit pyrogenicity test for bacterial endotoxins, but not generally measured in viral tests. But, fever may contribute to feelings of malaise that may manifest in observable behavior and can be indirectly monitored in this fashion.

Tumor Formation

Although not used routinely for adventitious virus testing, in certain cases, regulators have asked for novel cell substrates to be assessed for viruses that might be oncogenic in vivo. Such substrates would generally be restricted to those that are derived from tumors or have been shown to be tumorigenic themselves in animals, causing the regulators to question what the cause of the tumorigenic profile of the cells may be. Could the tumor have arisen from an oncogenic virus infection? In assessing for oncogenic viruses, generally the regulators would ask for in vivo testing of cell lysates (lysed to release the purported virus) or cellular nucleic acids (to detect infectious viral genomes of oncogenic viruses). Unfortunately, these tests are currently neither validated, nor controlled to demonstrate validity. However, work has been published on a sensitive model and a positive control for assessing cellular DNA oncogenicity (Sheng-Fowler et al. 2010; Sheng et al. 2008), and discussed in September 2012 at a Vaccines and Related Biological Products Advisory Committee (FDA/CBER/OVRR 2012a, b). As a consequence of the lack of validation, these tests are not routinely employed.

Nonetheless, the concept is the following: were infectious oncogenic viruses or viral nucleic acids present in a cell substrate, they could cause the animals to develop tumors either at the site of injection or at remote sites where the virus may have circulated upon infecting the test animals. These tests require monitoring the animals for much longer periods of time than routine adventitious agent tests and to palpate the animals to detect nodule formation. Histopathology of animals that develop nodules on study and of those that do not by the time of the study endpoint may permit detection of occult lesions or metastases. Improved animal models and the availability of a positive control that will not infect animals and contaminate an animal facility may see such methods increase in use in future. However, the value of such a test in comparison to other novel methods that are emerging will need to be considered prior to routinely implementing a product safety test that requires the

use of animals given the current climate in which the reduction, refinement, and replacement (3 Rs) of use of animals in product safety testing is being staunchly advocated.

Antibody Production

Animals inoculated with a specimen contaminated with viruses for which that species is susceptible may mount an immune response to that virus contaminant. These antibodies may be detected by use of an IFA or ELISA assay. In this way, specific viruses can be sought that are relevant to a particular species, e.g., hamster viruses that might contaminate Chinese Hamster Ovary (CHO) cells, which could be used to produce recombinant subunit vaccines. This is the basis for the HAP, RAP, and MAP tests, described in Sect. 10.2.2 above. Because these tests are for specific viruses, they can be replaced with other specific methods, e.g., PCR. Regulatory acceptance of these alternative tests is emerging, e.g., by the Office of Vaccines Research and Review at FDA. Acceptance of alternative methods is dependent on demonstration that they are equivalent or better than the traditional methods. A focus on relative sensitivity (LOD) and specificity (e.g., lack of interference of test sample matrices) are key to international convergence for replacing these animal-based assay methods.

10.2.3 Challenges with Currently Routine Tests

There are a number of challenges and difficulties faced when employing the currently routine suite of viral tests.

Like all assays, false positives may result, leading to investigations and decision-making processes about whether a re-test is appropriate.

In the tissue culture tests, it is not infrequent that apparent positives can be linked to cross-contamination from the assay positive control virus. The animal-derived serum used in the culture media can also, sometimes, be a source of contamination of the assay, leading to a false positive for the specimen tested. Sometimes, cell monolayers do not maintain well over a 2-week interval required, leading to inability to assess them adequately for viral CPE or giving a false impression of viral CPE. Test articles can be cytotoxic, also interfering with the test and giving inconclusive results. Occasionally, a "bad" lot of RBCs will result in very high background levels in the hemadsorption portion of the test, which appear positive or inconclusive.

PCR tests, being so highly sensitive, are also subject to a false positive rate. Test articles frequently interfere with the PCR reaction, requiring dilution of the test article to overcome the inhibition, thus reducing assay sensitivity. And as previously discussed, PERT assays can be subject to false positives or high background

from cellular DNA-directed DNA polymerases or authentic RT expressed from endogenous, noninfectious retroelements.

The in vivo assays are also fraught with challenges. A poor or inexperienced dam may not suckle or tend her newborns properly, leading to a loss of a part or all of a litter. Or the pups that die may be cannibalized by the dam, precluding investigation into the cause of their deaths. Eggs can become bacterially infected and die from this, having nothing to do with the test article being contaminated. Or the test article may be toxic to the eggs. Even the adult mice can occasionally spontaneously die from unclear causes. If housed together, sometimes they fight and one may die from this pestering. All of these events can cause the appearance of a false positive or invalidate the test, leading to re-tests and loss of confidence in the results.

Currently, the test methods in different regulatory regions are not completely harmonized. The volumes, the routes of inoculation, the age of egg embryos at inoculation, length of incubation, and other differences exist. Also, due to lack of specificity or clarity in the various requirements and guidances, differences in the tissue culture tests also exist. The impact of these differences on the sensitivity and specificity of the methods is unknown, because these tests have not been validated as newer methods are required to be. They are considered compendial and need only be verified. Thus, the true performance parameters of the methods are relatively unknown. Some work (Gombold et al. 2014) has been done to address this problem, but it is only a beginning and does not provide comparisons between various compendia and regulations, having only followed the US methods.

Other specific challenges are discussed below in more detail.

10.2.3.1 Neutralizing Antisera

It may be necessary to neutralize the vaccine virus in order to perform the test for viral adventitious agents in the panel of indicator cell cultures or in vivo systems, because the vaccine virus might replicate in the test system or may just lead to a cytotoxic defense response, in the case of some replication-defective viral vectors. Although this issue may be addressed during vaccine production by the use of control cells, the need to test the viral seeds or pre-seeds to demonstrate that the input material into production is free from adventitious agents makes this issue problematic to address. The cytotoxic response that may be caused by inadequately neutralized vaccine virus might lead to complete cell death or a subpopulation of cells might recover after some period. In the latter case, a judgment needs to be made as to whether the test could be considered valid if a large proportion of the inoculated cells died. Incubation of the vaccine virus harvest sample or seed with neutralizing antisera raised in animals or specific neutralizing monoclonal antibodies can alleviate the potential for viral replication, and might be able to alleviate cytotoxic response.

Neutralization can be challenging however. The antisera used must be “completely” neutralizing, because any vaccine virus not neutralized may break-through, infecting the test system and causing positive results in the assay. Completely

neutralizing antisera cannot always be raised against some viruses. For example, pox viruses are very difficult to neutralize completely and this presents problems for testing for adventitious agents of vectored vaccines based on pox viruses. Testing parallel control cells addresses some concerns, for adventitious agents that may have arisen from the production cells, the culture media components, or the production process itself (equipment, environment, personnel). However, this does not address adventitious agents that may have arisen from the viral seed or the species from which the isolate was derived, if not molecularly derived or cloned.

The species in which the neutralizing antisera are raised should not be susceptible to viral infections that may be adventitious in the production system, or other antibodies not specific to the vaccine virus may be present in the antisera and risk neutralizing the adventitious viruses one is trying to detect. Determining that interfering antibodies are present in antisera is not technically feasible, as one cannot know all the adventitious agents against which one might need to screen the antisera.

Consequently, there is an advantage to using monoclonal antibodies or raising antisera in Specific Pathogen Free (SPF) animals. SPF does not mean being free of all pathogens nor of nonpathogenic (in that species) viruses, but only means being free of specific pathogens, as the term implies. Also, the degree of "SPF-ness" can vary, in that one can have differing numbers of pathogens from which a herd, flock, or colony of animals must be free. So, for different purposes, one may have a list of, e.g., 10 pathogens, 15 pathogens, or 25 pathogens, for which a herd, flock, or colony is monitored. All of these would be considered SPF, but some would be "more SPF" than others. Also, the list of specific pathogens may not be harmonized across regulatory regions because of different viruses endemic in various regions (which presumes the tests are being performed in the same region as the regulators who are reviewing the test data, which is not always the case).

Other concerns with use of a neutralizing monoclonal antibody or antiserum include the small dilution of the test sample, thus slightly reducing the sensitivity of the test. Also, there may be potential toxicity for the test system. In the latter case, reducing the antiserum concentration to nontoxic levels, while maintaining sufficient levels of neutralization to prevent break-through, may be a fine line that might not be reliably achievable.

All of these issues must be borne in mind when testing viral seeds and vaccine harvest material for adventitious agents in the test systems of living organisms and cell cultures. Each can complicate the reliability of test performance, or in some cases, even preclude it.

10.2.3.2 Dose Equivalents and Test Samples/Volumes

Unlike the specificity of methods for sterility tests, the various compendia and regulations have not always been clear regarding the amount of test article that should be applied to the test system. Different testing service providers apply differing amounts and even between clients, they may receive different

concentrations and volumes of test sample. While the volumes for the in vivo tests are specified, the concentrations of the material applied are not always clear. In FDA regulations that were revoked in 1997 as being obsolete, restrictive, duplicative, or unnecessary,[1] the test methods were described in the context of testing of viral harvests of specific vaccines and so dose equivalents were given in terms of viral titers that would reflect a final container dose for that vaccine. The origins of the specific guidelines are very likely rooted in what was considered practical at the time the regulations were first promulgated. Cornfield et al. (1956) described the application of 500 dose equivalents for detecting residual infectious poliovirus after inactivation in order to provide a 1/100,000 chance (after multiple tests at different process stages) that any given dose might contain an infectious unit of poliovirus. Likewise, 500 dose equivalents (or minimum volume of 50 mL, whichever was greater) was promulgated in the 21 CFR-mandated testing for measles, mumps, and rubella vaccines in the 1960s, although the concept was entirely different since these were live vaccines, and the tests were for adventitious agents rather than residual live vaccine virus following inactivation. The similar figure of 500 dose equivalents was not statistically derived, nor is there literature describing the probability of detection of an adventitious agent in a dose of vaccine, as Cornfield et al. did for inactivation of poliovirus. Similar to the revoked U.S. regulations, the European Pharmacopoeia (EP) section 2.6.16 (EP 2014a) specifies testing the greater of 50 mL or 500 dose equivalents for both virus seeds and harvests. However, for testing cells, cell lysates, spent culture fluids, or viral seeds (in the case of the U.S., although this is addressed in the EP as stated above), there was no clear guidance. The FDA guidance document that was finalized and published in 2010, "Guidance for Industry Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications," made an effort to add clarity on the recommended amounts to test for these samples (FDA/CBER/OVRR 2010).

The recommended inoculation routes and volumes do not necessarily assure a consistent sensitivity for all potential adventitious agents that might be detected by a given method, and actually reflect practical capabilities scaled linearly by replicating flasks or animals. For instance, intracranial inoculation of 0.01 mL of a culture fluid into each of 20 suckling mice is unlikely to yield a comparable volumetric sensitivity for the neurotropic virus LCMV than testing 0.5 mL of the same fluids in each of 10 or 20 eggs does for an influenza virus. Similarly, testing 100 dose equivalents in eggs is not the same sensitivity as 500 dose equivalents in cell cultures. Testing 10^7 cell equivalents as part of cell substrate characterization in animal and cell culture systems is unlikely to represent the same sensitivity as

[1] The reason the regulations were revoked was because the conditions promulgated in the regulations were contained in the licenses for the specific products (viral vaccines against polio, measles, mumps, rubella, smallpox) to which the additional standards applied. In other words, they were deemed duplicative and unnecessary, because the standards were addressed in each specific license and no longer needed to be addressed in regulations. Hence, the test methods are still applied, even though the regulations were revoked.

testing the maximum nucleic acid load per well in a PCR assay for a specific virus (typically ~0.5 ug, representing roughly 10^5 cell equivalents). Consequently, even among the existing routine tests, the same level of sensitivity is not expected for each method and the sample volumes tested reflect the practical needs of the method rather than a statistically determined sample, which would be the ideal approach.

Finally, lack of contamination cannot be completely assured unless the entire batch or lot is tested in all suitable assays—an obvious impossibility. In fact, all sampling strategies are a trade-off between how much material can be sampled and the concentration of the contaminant that can be detected with that amount of sample. Even the strategies described above do not assure absence of potential contaminants or risk, but rather provide a level of assurance that, in the context of validated or qualified manufacturing processes, there has not been a catastrophic failure in the system. This is analogous to the relatively small volumes of material tested in the compendial "sterility" tests, which only support product sterility by indicating that there has not been a catastrophic breach in the validated sterile processes. A corollary to this assertion is that low level contaminants or non-homogenously distributed contaminants are unlikely to be detected in the routine tests. For this reason, testing should not be the sole basis on which to assure product biosafety. Appropriate sourcing and quality control of raw and starting materials, adherence to Good Manufacturing Practices, environmental and personnel monitoring, process validation, and finally, testing as verification are the package needed for maximal assurance of biosafety. We address the inherent and necessary connection between process and testing in the context of viral safety margin in Sect. 10.3.

10.2.3.3 Is Anything Missing in the Current Methods?

Besides the obvious answer of yes, it must be noted that the vaccine industry has successfully relied upon the current suite of tests for decades now. Although some viral contaminations have gone undetected (e.g., infectious porcine circovirus in rotavirus vaccine), these examples are few and far between. Most adventitious agents are detected prior to release of product into the clinic or onto the market, and often even before downstream processing has occurred, or at the stage of cell substrate or viral seed qualification, before production has even begun. In the era of cGMP (since the 1970s) and use of well-characterized cell banks for production (since the 1980s), viral contamination events are relatively uncommon. Nonetheless, they are inevitable due to the biological platforms used for manufacturing, and continue to occur despite scrupulous measures to avoid them. Thus, testing strategies must continue to account for newly emerging threats, as well as the commonest of the, albeit uncommon, contamination events.

Further, it should be acknowledged that the current general screening testing methods do not detect numerous animal viruses that exist in nature. As noted above,

Table 10.1 Viral families and their potential to be detected by the indicated test methods

Virus Family	Embry-onated eggs	Adult and suckling mice	Guinea pigs and rabbits	Routine *in vitro* cell cultures
Adeno-				
Arena-				
Arteri-				
Astro-				
Bunya-				
Calici-				
Circo-				
Corona-				
Filo-				
Flavi-				
Hepadna-				
Herpes-				
Orthomyxo-				
Papilloma-				
Paramyxo-				
Parvo-				
Picorna-				
Polyoma-				
Pox-				
Reo-				
Retro-				
Rhabdo-				
Toga-				

specific PCR tests fill some gaps, when deemed relevant. Many of these viruses are incapable of propagating in cell culture, thus unlikely to be present after viral vaccine strain development and vaccine production; thus, they have been safely "ignored" after due consideration that they did not pose a viable or significant threat. Table 10.1 illustrates the expected capabilities of the existing routine tests to detect or not detect important representatives from families of viruses, based on a good faith review of diagnostic virology literature—green indicating a generally suitable combination, yellow suggesting either limited applicability or need for unique conditions, and red indicating generally not considered suitable for detection (viral families appear alphabetically).

The porcine circovirus example is illustrative. The virus is not readily detected in conventional tests, and therefore was able to propagate in a cell substrate without notice. Specialized tests for porcine circovirus might not have been requested previously, since it was probably considered unlikely to propagate in manufacturing substrates, and was not known to be pathogenic to humans. However, while not a significant safety concern, purity of vaccines must also be considered when thinking about adventitious agents. What may appear to be safe may still not be pure or suitable. The PCV event made it clear that regulatory agencies do not want *infectious* adventitious animal viruses in vaccines (consistent with language about "demonstrable viable" viruses in previous regulations), although remnants of inactivated organisms may be present and may be tolerable as impurities.

Some of the gaps in coverage by the tests listed in Table 10.1 are covered by recommendations for specific PCR and in the case of retroviruses, by a PCR-based reverse transcriptase assay. For instance, neither Hepatitis B nor C propagate in cell culture, and HIV requires particular kinds of cells, which require nonstandard culture media supplements, such as IL-2, to propagate well in culture, or specialized engineered cell lines with appropriate receptors. These culture conditions or specialized cell lines are not reflected in the current tissue culture tests. However, because of the severe impact of such potential contaminants, even the extreme unlikelihood of their presence or inability to propagate in most cell substrates has been deemed by regulators as an insufficient rationale to not test for them, in appropriate settings (e.g., when human cells are used in production). This point introduces one of the concepts of risk assessment discussed in greater detail in Sect. 10.3.

Another issue that challenges any viral test method is that viruses are so highly variable. Variants may occur that are not detectable by a specific test, even though most variants or strains are detectable. Strain differences and even single nucleotide mutations can result in changes in tropism (susceptibility of test system to infection) or viral fitness (ease of infection and/or replication), as well as pathogenicity (readout in in vivo tests and concern for human recipients). Each of these types of changes can result in variants to which a particular test system may become refractory or lose sensitivity.

This ability of the test systems (cell lines or animals/eggs) to detect infections can be affected by species barrier, tissue-specific tropism, possible need to adapt to the culture system, and whether the readout would actually reveal the contaminant, if it was present. For instance, both SV40 contamination of primary macaque kidney cells and PCV contamination of a Vero cell substrate were not revealed as infectious contaminants until tested on either a different cell substrate (SV40 on African green monkey kidney cells) or with a different method (for PCV, using modern genomic testing). In the best case, however, the cell culture and in vivo methods can potentially detect viruses that are not known today, but may emerge in the future, as long as they can infect the systems and produce the same readouts as viruses we currently know (i.e., cytopathic effects, hemagglutination/hemadsorption, death, overt illness, pocking).

Existing molecular methods cannot be taken for granted either. Viral variants are common, and have the potential to escape detection if their sequences do not closely match those for which molecular tests were designed. At the very least, molecular methods should be reviewed regularly to assure coverage of the most recent viral sequences. In the best case, primers and probes developed against conserved regions might be relatively resistant to some of the viral variation, allowing detection of novel strains of viruses that share those conserved regions with known strains.

Another issue that challenges the more historical methods is that they have not been subjected to systematic assay validation as the International Conference on Harmonisation and Pharmacopoeia recommend or require for modern assays—and arguably cannot be validated for all agents for which they might be susceptible. Typical verifications of the compendial methods for cell culture-based assays, for instance, might utilize only a few viruses (not unlike qualification of sterility tests), and in vivo assays have never, until recently (Gombold et al. 2014), been challenged systematically to our knowledge. Compounding the lack of validation of certain conventional methods is the variety of cell and animal strains being used, the variety of culture conditions or inoculation and incubation conditions, and lack of widely accepted standards with which to establish performance parameters (for limit tests, primarily sensitivity and specificity).

The existing assay methods remain largely unharmonized between the major regulatory regions (e.g., U.S., Canada, EU, and Japan) in terms of exact details of how to perform the tests. The impact of small differences on assay performance is unexplored, such as the inclusion of additional routes of inoculation in the in vivo systems, which one would think could improve the sensitivity, but may paradoxically result in interference, and thus diminution in the sensitivity or specificity. Likewise, reducing the volume inoculated into eggs or changing the age of the embryos at the time of inoculation could be seen as potentially reducing the sensitivity, or improving it, by reducing toxicity effects from the test article. The impact of such test variations on assay performance is unclear and unknown, because the assay performance for any of these methods are generally unknown.

Finally, as new production systems are explored or incorporated into a license for new vaccines, challenges to detect unique adventitious agents will arise. For instance, should we worry about plant viruses or most insect viruses (beyond those that cause vector-borne infections in humans)? Arguments about previous exposure to plant viruses via foods are obviously inadequate since many medicines are injected and therefore bypass natural immune mechanisms. Arguments about potential for recombination and unanticipated consequences can seem theoretical at best and quite speculative at worst. If regulators and manufacturers are operating in an information vacuum, it will be difficult for them to say there is no cause for concern. Furthermore, while plant or most insect viruses, for instance, may not seem like safety issues to human recipients or potentially capable of giving rise to emergent human pathogens, they nonetheless remain an impurity concern. They also have the potential to negatively impact the manufacturing consistency of plant- or insect-based production systems, just as MVM or vesivirus 2117 contaminations

have negatively impacted manufacturing of CHO cell-derived therapeutic proteins, causing lengthy and costly facility shutdowns and remediation, and even product supply shortages of important medicines. The current suite of tests, with the exception of TEM, are essentially incapable of detecting plant viruses, and many insect viruses would also be missed unless specific PCRs are incorporated. TEM is the notable exception and in fact, an insect virus contaminant that had been observed in a production insect cell line, was detected by careful evaluation of micrographs of an unusually large number of cells than would typically be examined. But, as this would not be done routinely, this approach could not be relied upon for this purpose.

So, in summary, the current suite of tests, though reasonably robust and largely reliable, have limitations and leave certain gaps and room for improvement when developing scientifically driven testing strategies.

10.2.3.4 Toward Global Safety Standards

Efforts have been made to harmonize viral safety guidance, but the major harmonized guidance [ICHQ5A(R1), International Conference on Harmonisation 1999] does not include viral vaccines within its scope. Major international guidance on viral safety applicable to viral vaccines is available from the US FDA (FDA/CBER/OVRR 2010) and WHO (Petricciani 2010); and from pharmacopeia including the European Pharmacopeia (EP5.2.3 for animal cell substrates, 2.6.16 for viral vaccine seeds and harvests) (EP 2014a, b, respectively), Japanese Pharmacopeia (2011), and likely other similar pharmacopeia from other countries. While the various regulations and guidances are relatively consistent, there are inevitable differences. The extent to which differences are accommodated when new products are registered or existing registrations are updated in different regions is not entirely clear. We cite a few differences here that are relevant to viral safety analytics:

- EP2.6.16 mandates use of control cells for viral vaccines, whereas FDA and WHO guidances suggest circumstances in which they might be useful, but do not mandate them for all viral vaccines. None of the guidances clarify what is recommended to be done for control cultures of suspension-adapted cell lines used to produce viral vaccines or vectors, or recombinant protein vaccines made in viral-vectored expression systems.
- WHO guidance and EP2.6.16 specify testing vaccine harvests and seeds for avian viruses using embryonated eggs, only if the vaccine is made in avian cell cultures or eggs, while FDA guidance appears to mandate testing in eggs regardless of the animal cell substrate used for manufacturing. Both EP 2.6.16 and FDA specify a 100-dose equivalent requirement for testing. EP5.2.3 guidance for cell substrates specifies testing any animal cell substrate in embryonated eggs, and there are minor differences in the method description compared with EP.2.6.16.

- EP5.2.3 and WHO specify 4-week observation of IP-inoculated adult and suckling mice, while EP2.6.16 and FDA specify 21-day observation of IP- and IC-inoculated adult mice (WHO recognizes the IC route in small print). Both FDA and EP2.6.16 specify an initial 2-week observation of suckling mice, but only FDA specifies a blind passage of tissues from surviving mice into another set of suckling mice for an additional 2 weeks (WHO recognizes the FDA option in small print). [The recent work by Gombold et al. 2014, suggests this blind passage does not result in enhancement in sensitivity of the test.]
- FDA, WHO and EP2.6.16 specify inoculation of guinea pigs (IP all; IC only FDA, but recognized by WHO in small print) followed by observation for 42 days to detect *Mycobacterium tuberculosis* and evidence of LCMV or other viruses. FDA allows for the test for *M. tuberculosis* to be replaced by validated in vitro culture and PCR methods (WHO allows shortened culture with PCR endpoint). It is unclear if the 42-day test would still be required exclusively to detect LCMV if another test is performed for *M. tuberculosis*. EP5.2.3 does not specify guinea pig testing.

Regulators in emerging markets appear to be adopting or adapting WHO or ICH/EP-like guidance or they might develop their own guidance. The development or reevaluation of standards in existing and emerging markets presents a unique opportunity to drive toward global standards for viral safety. This is in keeping with the WHO's position that whether a vaccine is manufactured in or for a developing country or a developed one, the minimal safety standards must be the same.

10.2.3.5 Innovation

FDA regulations permit substitution of new tests for existing ones per 21 CFR 610.9 (Code of Federal Regulations 2012a), which states that doing so is only permissible when there is evidence that the assurances of the safety, purity, potency, and effectiveness of the product provided by the new method are "equal to or greater than" the assurances provided by the old method or the compendial method. This regulation does not clarify how to go about producing such evidence, but only permits it to be done.

If the evidence can be provided using the same units of measure or if head-to-head comparisons of the methods can be made, it is more straightforward how to develop the required evidence. Sensitivity of the existing cell culture-based adventitious viral tests is defined in terms of an infectious virus input, which is typically qualified by means of spike recovery studies for unique test article matrices. Thus, the LOD is defined in number of plaque-forming units (PFU) or TCID50 (tissue culture infectious dose that infects 50 % of wells in a cell culture assay), i.e., infectious units. Rules around ethics of animal usage and simple practical concerns preclude routine qualification of animal-based tests by infectious virus spike recovery studies.

In contrast, for some of the newer methods, e.g., PCR or any of the methods that detect viral nucleic acids, the sensitivity may be reported in genome copies/reaction or per volume of test article, or some other similar measure. Most viral preparations contain large numbers of noninfectious or defective viral particles in addition to the infectious ones. Those defective particles may contain nucleic acids, but not contribute to propagating an infectious contamination. In fact, residual detectable nucleic acids may be present even when all infectivity has been neutralized or inactivated, or when the viral preparation is intentionally of replication-incompetent or defective viruses. Therefore, there would be no consistent or clear-cut concordance between a quantity of nucleic acid and an infectious unit.

This same problem has challenged the efforts to compare a PCR-based method for detecting mycoplasmas with the standard tests. Some efforts have been made to develop standard reagents that are controlled for the number of genomic copies to infectious colony forming units in order to facilitate comparison and to validate the relative sensitivity of the methods. Might a similar approach be considered for viruses?

10.2.4 Emerging Analytical Capabilities

New capabilities are being developed to detect viruses or their components (e.g., nucleic acids) in response to a variety of needs. Among these needs are the recognition of new or emerging potential threats, recognition of the limitation of existing methods, need for results in less time to support both some new types of products as well as to enable rapid response to actual contamination events, and a desire to reduce or replace animals used for product safety testing. Arguably, much of the impetus and funding for new technology development in the last decade has been related to biodefense and rapid characterization of emerging disease threats. Nonetheless, those involved with biosecurity, public health, clinical diagnostics, and biopharmaceutical adventitious agent testing share some mutual interest in breadth of detection, rapid turnaround, cost control, and where possible, simplicity, and robustness in use.

Biopharmaceutical applications are demanding in terms of compliance issues, bridging to existing methods, and implications of results. True positive results from novel methods have to be evaluated for their implications on safety of products that could be administered to the most vulnerable populations, and both true and false positive results have the potential to interrupt supply of critical life-saving medicines.

This section addresses opportunities for advancement in both existing and emerging methods. Some general issues are presented as well as alternative approaches for implementing novel methods.

10.2.4.1 Improving Culture-Based Detection by Improving Conventional Readouts

We begin by only briefly acknowledging some opportunities to improve on the current cell culture-based assays, which might arguably include standardizing the readouts, improving time to detection, and/or enabling sensitivity to viruses that are not otherwise readily detected.

One approach to improving the current culture-based assays would be to standardize the microscopic visual readouts (CPE, hemadsorption, immunofluorescence) by use of machine vision coupled with pattern recognition algorithms. Standardization could reduce analyst-to-analyst variation, reduce the training burden (especially in high turnover laboratories), and potentially increase throughput while reducing labor costs. Rather than spending labor time reviewing the overwhelming proportion of normal (negative) cell sheet surface, labor time could be focused on verification of the much smaller proportion of questionable features in cell monolayers identified through pattern analysis algorithms. But despite progress in imaging, robotics, and analysis, off-the-shelf systems might not currently suffice for broad application in adventitious virus quality control testing. Assembling custom systems that have this capability presents formidable challenges, including but not limited to complexity of automation, rapid acquisition time, depth of field and focus (and thus clarity) in plastic vessels of differing dimensions, image storage and analysis time, and compliance with 21 CFR Part 11 (Code of Federal Regulations 2012b) requirements for computer-based systems. The business case is challenging as well, given the cost and time that would be required for implementation, the variable track record for successful automated image-based applications in quality control, the rapid obsolescence of technologies in the context of license/marketing authorizations in which details of testing are documented, the possibility of only limited reduction in laboratory FTE requirement, and perhaps also the fact that the diagnostic virology community is moving toward more convenient and often nucleic acid-based measures of viral infection.

Another approach to improving existing methods for virus detection might be use of customized cell substrates or culture conditions. There are numerous descriptions of reporter cell lines developed for specific viruses (a few examples, HSV, CMV, VZV, BIV, Herpes B virus, alphaviruses, influenza, rubella) usually developed for the purpose of investigating or verifying specific viral infections. However, useful for specific viruses in clinical settings, the promise envisioned in an excellent review (Olivo 1996) has not benefited biopharmaceutical testing. Others have considered recombinant cell lines overexpressing antiapoptotic genes or primary cells treated with apoptosis inhibitors as means of enabling greater viral replication, and thus enhancing the detectability of transforming viruses (Sandstron and Folks 2001 and references therein). Improved sensitivity or breadth could also be accomplished by choice of cell line as well as selection of clones with better assay characteristics. For instance, Gombold et al. (2014) demonstrated differences among cell lines used in conventional assays, suggesting for instance that HeLa and A549 cells were more sensitive than MRC-5 and Vero for adenovirus 41 and

rhinovirus, although the A549 cell line was dramatically more sensitive than HeLa for adenovirus type 5. One company serving the diagnostic virology community commercializes prepared cell lines and mixtures of cell lines for more rapid detection of specific categories of viruses. Leland and Ginocchio (2007) reviewed many of these improvements relevant to the clinical diagnostic laboratory.

Few, if any, of these emerging capabilities have proven practical for biopharmaceutical adventitious agent testing. Conventional biopharmaceutical testing laboratories may not be inclined to take advantage of the more rapid readouts for narrower ranges of potential viruses because of the considerable increase in logistical complexity and cost of managing additional lines with only marginal added scientific value. Furthermore, the cost and complexity of qualifying numerous additional cell lines would represent a barrier to their implementation. Finally, an improved indicator cell line might well be suited to more rapid detection if the virus is present, but the duration needed to confidently call a result negative might not be easily changed from the 28 days now typically required. The fact that the vast majority of tests would result in negatives means that these approaches might not really improve the speed at which material might be released to the market, clinic, or for further manufacture. And the increased number of cell lines used can increase the potential for false positives, leading to re-tests and lengthy investigations, actually delaying release, without necessarily enhancing safety.

10.2.4.2 Advances in Alternative Detection "Readouts"

Several technologies are opening the possibility of expanding the breadth of detection beyond that of the conventional tests, even encompassing all known and even possibly as yet unknown viruses. This possibility is so compelling that we are forced to ask whether these new methods are capable of providing greater assurance of biological safety than the conventional methods. Certainly in terms of "specificity" (i.e., breadth) they could, but whether their level of sensitivity would be adequate needs to be explored. There are data to suggest that some of these readouts might be less sensitive than a specific PCR, but the breadth is clearly far greater.

The advances that will be considered here are perhaps best described as alternative approaches to detecting viral nucleic acids or transcripts, and viral proteins. Rather than attempt a comprehensive review of the extensive scientific and commercial literature, we will attempt to capture the essence of the technologies that have shown (or arguably could show) promise in applications closely related to biopharmaceutical testing and explore principles that will be essential in their standardization and qualification for regulated testing. A more detailed review of some of these methods is being prepared by a task force of the Parenteral Drug Association, for publication in 2014 and in proceedings to be published by the PDA of a conference held in Nov. 2013 on advanced detection technologies. Some of these methods are already being used for assay investigations and as part of viral risk assessments.

Next Generation Sequencing

Today, there are several mature and emerging platforms for nucleic acid sequencing for which excellent reviews are available (Niedringhaus et al. 2011; Glenn 2011; Metzker 2010; see Kolman and Onions this volume). The so-called "next generation" sequencing technologies differ from classical DNA sequencing by making available the individual nucleotide sequences of every template fragment analysed, rather than a single most represented sequence of a population of fragments. Next generation sequencing platforms differ in the "read" lengths they generate, the practical depth per nucleotide, inherent error rates, cost, turnaround time, and flexibility for other analyses. For instance, de novo assembly of reads is arguably easier with reads that offer longer potential overlapping sequences. Thus, the sequencing technology chosen for the purpose must be considered in terms of its capability to meet the needs of the intended use, as the differing technologies have different capabilities.

Generation of the raw sequences for a population of fragments is only the first step in the use of this technology to detect and identify potential viruses. Analysis algorithms are applied in which the sequences might be directly searched against viral databases, or further processed to improve quality of "hits" (i.e., by de novo assembly and/or translation in all reading frames prior to searching). The particular sample might contain cellular DNA and/or RNA, which affects both sequencing and analysis. The more nonviral nucleotides that are present, the more sequences that must be generated and analysed to detect the potential viral sequences.

So approaches at the level of sample preparation and/or data analysis may have to be incorporated to either minimize these sequences (e.g., ribosomal RNA) or subtract signals from the population of sequences. However, an arguably better approach is to positively select potential viral sequences by matching against databases, which might require more sequencing and computational effort, but reduces the chance of systematically eliminating potentially meaningful sequences.

Once lists of virus accession hits are generated, these must be triaged, if the viral database has not been rigorously curated. For instance, it is common to hit certain viral accessions that also contain cellular (nonviral) sequences. If only the nonviral sequences were hit in such a database accession, these can be regarded as false positive hits. Evaluating the relevance of hits also requires considering the depth and coverage of reads. Very narrow coverage of a viral gene or genome might be explained by residual nucleic acid, for instance from expression vectors used to make some of the biological reagents used in the method. Coverage of a full viral gene or genome might be consistent with a viral transcript or intact virus. Inferring biological relevance to positive hits depends on the sampling scheme, sample preparation, and suite of controls; often additional and orthogonal methods are needed to evaluate potential positive results. Thus, extensive bioinformatics and virology expertise are needed to process the data.

Sequencing approaches that do not rely on predefined targeted primers are relatively unbiased in the sequences that can be generated. If identifications are only accomplished by matching to existing viral databases, however, there is some

potential bias contributed by the breadth and depth of the database. Truly novel sequences that do not match well against the existing database might not be recognized as representing a novel virus unless they are assembled without scaffolding against an existing viral reference sequence. Thus, the unbiased potential of next generation sequencing is only realized when the possibility of novel viral sequences in unmatched read population is addressed. The turnaround time, in our experience, for a study from extracted nucleic acids to final analysis has typically been at least several weeks.

Next generation sequencing has been used effectively to characterize live virus vaccines for potential unexpected viral and microbial sequences (Victoria et al. 2010) and is now available as a commercial service specifically for virus detection at contract research laboratories. Next generation sequencing has been applied after other similar sample preparation strategies (de Vries et al. 2011) as well as after highly multiplex amplification of numerous potential viral targets (Hall et al. 2012).

Efforts will be needed toward standardization of databases and viral spikes for assessing sensitivity, as well as data-sharing in order for practitioners and regulators to converge on the most meaningful sample preparation and analysis strategies.

High-Density Microarrays

Detection/identification microarrays use oligonucleotide probes designed to hybridize with known viral sequences, typically at multiple sites across the viral gene or genome. The probe design operation is performed for as many viruses or viral sequences as desired—the highest density chip to date covers all sequenced viruses, bacteria, and fungi and incorporates ~388,000 probes (Munroe 2011). Amplification strategies have been applied prior to array analysis to enhance sensitivity (Erlandsson et al. 2011). In contrast to de novo sequencing, the bioinformatic analysis of arrays is essentially performed prior to ever running a sample on the array—in the probe design phase. After a sample is actually run on the array, an analysis algorithm calculates the probability of a positive hit for viruses based on factors like signal intensity, coverage across multiple targets within the same virus, and hits to closely related viruses. Access to such arrays is now available on a fee-for-service basis from at least one commercial testing laboratory. The turnaround time from the random amplification/labeling reactions to final results can be about one day.

Re-sequencing arrays are designed with a narrower goal—that is to confirm the nucleotide sequence of a narrower range of viruses or viral genes. Importantly, their objective is to evaluate the sequence of targeted regions by using probe sets that present, for one base position at a time, all four possible bases at that position. These arrays are typically designed for pathogens of interest, for instance in biodefense (Leski et al. 2011) or specific public health situations (Berthet et al. 2010) where detailed sequence information is needed very rapidly in order to formulate a response. The utility of these types of arrays for adventitious agent testing, where the goal is detection, is less clear, though they may be useful in manufacturing

investigations. Such investigations are triggered when an adventitious agent has been detected. Investigations are an important component of quality assurance to aid in identifying the source of a contaminant and suggest potential corrective and preventive actions, and in this manner, arrays may be useful for biologicals production.

Mass Spectrometry (MS)

Protein mass spectrometry by MALDI TOF has been quite effective in identifying bacteria biologically amplified in culture and even from clinical samples (Wieser et al. 2012). Improved workflows are even enabling liquid chromatography (LC)-MS/MS-based strain typing (Karlsson et al. 2012). Application to detection of viruses, however, is limited by sensitivity and complexity of typical viral samples. Recent reviews of progress in proteomic analysis of viruses and virus-host systems demonstrated the increasing capability of MS combined with separation techniques in rapidly characterizing viral and virus-host interactions from relatively complex samples (Zheng et al. 2011; Zhou et al. 2011). Despite advances in sample preparation and analysis, proteomic studies have focused on a relatively small number of specific viruses—HCV, dengue, HIV, influenza, SARS, RSV, and a small number of others, as reviewed in Zheng et al. 2011 and Zhou et al. 2011. The studies these authors reviewed were primarily directed at understanding pathogenesis and biomarkers of infection and other characterization of known viruses, rather than detection and identification of unknown viruses in samples.

Some investigators are, however, exploring the detection of unknown viruses in complex samples as a potential diagnostic tool. Ye et al. (2010) detected vaccinia proteins in cultured human lung fibroblast cells infected with an unidentified viral culture isolate when the infected cultures exhibited $\sim$60 % CPE. Sample preparation included detergent lysis and clarification of a supernatant fraction from cell pellets, cleanup, and protein separation by either 1-D or 2-D gel electrophoreses, cutting out bands or spots of interest, in-gel digestion, and analysis of peptides by LC-MS/MS. The authors also explored use of multiple proteases for in-gel digestion, which increased coverage of proteins detected from the 2-D gel preparations to as much as 89 %.

Konietzny et al. (2012) recently demonstrated the first detection of BK viral proteins from urine by LC-MS/MS tandem mass spectrometry, though after what was described as a slightly complex differential centrifugation/ultracentrifugation/filtration protocol to enrich for viral proteins. Observed peptide sequences were searched against a customized protein database. The method distinguished subtypes that could correlate with differing clinical significance. Importantly, algorithms are being developed to analyze the complex data from MS-based studies of complex samples, even whole viral digests, particularly for influenza: FluAlign and FluGest (Schwahn et al. 2009); FluTyper (Wong et al. 2010), and FluShuffle and FluResort (Lun et al. 2012). Application of proteomic approaches to viral *detection* will depend primarily on sample preparation workflows and separation techniques to

improve sensitivity. It is less likely that hardware and analysis algorithms will be rate-limiting to this application. Other factors likely to affect wide adoption, as the technology matures, include initial cost of systems and expertise to run and manage them.

In contrast to the MS-based proteomic approach, analysis of short regions of nucleic acid sequences specifically amplified from samples have been very successfully applied to detection and identification of viruses and other agents (a few recent articles include Chen et al. 2011a, b; Deyde et al. 2011; Sampath et al. 2012). This technology is commercialized and finding some application in the biopharmaceutical arena (Sampath et al. 2010). The database against which observed amplicon masses are compared is proprietary, but is curated from public databases. This approach can be used for viruses, bacteria, fungi, and mollicutes. The claimed sensitivities, as reported in the literature as limits of detection (LOD) of the method, are somewhat dependent on the matrix of the specimen. The claimed LODs are in the range of 100–10,000 copies/mL for viruses (Chen et al. 2011b), on the order of 1,000 copies/mL for bacteria and fungi (Ecker et al. 2010), and as low as 5 copies/mL for mollicutes (Lawrence et al. 2010). The turnaround time for analysis from extracted nucleic acids to result can be about one day.

Other PCR-Based Methods

A variety of additional PCR-based methods have been evaluated for detection and identification of narrower subsets of viruses. Several multiplex approaches for detection of respiratory pathogens were reviewed in the context of clinical diagnostics, each with different mechanisms to detect the amplified signal (analogous to the ESI-MS approach noted above, Callendo 2011). These methods are narrowly scoped for a specific clinical application. A novel approach to very highly multiplexed PCR, with a next generation sequencing readout, has been demonstrated (Porreca et al. 2007; Li et al. 2009; Kozal et al. 2012). This technology has been developed as a means of enriching the population of sequences for targets of interest. Since the targeting oligonucleotides are short (<100 bp, typically), must be designed based on known sequences, and would need to be tiled across at least numerous conserved targets in viral genomes of interest, this strategy would share the sequence specificity and design considerations of both conventional PCR and microarrays, coupled with the turnaround time and analysis considerations of next generation sequencing.

An alternative approach, using degenerate oligonucleotide primers without prior sequence knowledge of the specific viral target, offers promise as a near-universal assay (Uhlenhaut et al. 2009). In this case, amplification used an oligonucleotide primer demonstrated to detect a range of viruses (Nanda et al. 2008), and discrete amplification products were isolated from gels, cloned into sequencing vectors, and amplified in bacteria. Identification was accomplished by conventional sequencing. This procedure could be challenging if there were numerous amplification products. Use of next generation sequencing as the readout after degenerate amplification,

instead of cloning and conventional sequencing, might be a compelling alternative (McClenahan et al. 2014). Its relative analytical performance in comparison to other broad detection methods needs to be established, and consideration needs to be given toward how a large panel of degenerate primers could be controlled and implemented for regulated testing.

Using Antibodies

Novel signal amplification approaches have been used with specific antibodies. For instance, oligonucleotides can be conjugated to monoclonal antibodies enabling PCR-based detection of bound antibody. This method detected rotavirus with $\sim$1000-fold greater sensitivity than an optimized ELISA, and with clear separation between positive and negative stool samples even after 10^4-fold dilution of the positive stools (Adler et al. 2005). This method has also been applied to detection of the pathogenic isoform of prion protein in bodily fluids, with a claimed sensitivity based on recombinant PrP spikes to 10 pg/mL (König et al. 2006).

Other approaches use antibodies as part of a separation system to enrich for specific viruses that can then be characterized by other methods. For instance, Chou et al. (2011) used a specific monoclonal antibody conjugated to magnetic nanoparticles to concentrate influenza particles from allantoic fluid of embryonated eggs, and detected them to a sensitivity of $\sim 10^3$ ID50 influenza virus per mL using mass spectrometry. However, given the narrow breadth of detection, these approaches as solutions may not be suitable for broader viral safety testing for biopharmaceutical processes. Consideration needs to be given to the sensitivity of prion detection in biologicals by this approach however.

10.2.4.3 Some General Issues for Emerging Detection Methods

An important aspect of analytical validation is the demonstration that the method is suitable for its intended purpose. Here we focus on a brief characterization of features we consider important in determining "fit" for biopharmaceutical applications of the nucleic acid-based detection technologies. Most important of these features is clarifying the difference between a method designed to detect and one designed to identify in the context of adventitious agent testing. Both are important, but detection is paramount for a limit test for impurities.

Detection Versus Identification

Especially in the context of the emerging methods, the difference between identifying and detecting adventitious viruses needs to be clarified. Validation requirements differ between which performance parameters must be characterized for an identity test or for a limit test (for detection of impurities) [ICH Q2(R1) Validation

of Analytical Procedures] (ICH 1996). An identification test must be specific—capable of distinguishing related viruses at a definable level of difference. But any identification assay also has an inherent and definable level of sensitivity, though when used exclusively for identification, an assay's sensitivity has not traditionally been required to be defined. In fact, identifications are typically performed only after isolating or significantly enriching the particular contaminant.

In sharp contrast, sensitivity is the critical attribute of a detection assay (limit test for impurities), although specificity also needs to be defined. As a result, assays to detect viral agents have historically tended to evaluate relatively larger volumes or amounts of test article to achieve greater sensitivity. A further complication for establishing comparability of new methods is that detection assays have historically been calibrated in functional units (infectious units per volume of virus preparation based on a cell culture or animal-based infection system). Functional units typically vary from one infection system to another, and units do not necessarily equate with (though should not exceed) total viral particles or viral genomes. Specificity is also a required attribute of a detection (limit) assay, and is arguably of greater importance for novel assays where detection is based on recognition of a specific nucleotide sequence or protein "signature."

Thus, when considering whether a novel method is suitable to replace or supplement an existing detection assay for adventitious agents, the sensitivity of the novel method needs to be considered. Some of the new methods which are inherently capable of identification also lend themselves to alternative sample preparation methods that can enhance sensitivity, making them arguably suitable as detection methods.

Preparation of Nucleic Acids for the Readout

Viral nucleic acids may be composed of DNA or RNA. Starting from the simplest approach, total nucleic acids from a sample can be isolated and introduced either directly or after amplification steps. If nucleic acids from production cells are extracted, then cellular nucleic acids (genomic DNA, ribosomal RNA, cellular transcripts) could predominate and potentially limit the amount of cell equivalents or sample volumetric equivalents that can be introduced into subsequent reactions. On the other hand, cellular DNA allows detection of proviral sequences, which may be desirable. Total nucleic acids, or total RNA or total DNA, can represent viral genomes or potential transcripts (in the case of RNA) that are being produced within the cell—whether or not encapsidated. Messenger RNA (transcripts) reveals viral gene expression within a cell, suggesting active infection. Enrichment for mRNA (albeit both cellular and some viral) can be accomplished by annealing to beads coated with short poly-T sequences, since most mRNAs are polyadenylated at the 3′ end. However, some virus families do not polyadenylate mRNA, so would not be recovered by this approach (notably Flaviviruses, Reoviruses, Bunyaviruses). Where RNA is isolated, it is converted to cDNA prior to hybridizations or amplification steps. Total nucleic acids are well suited to detection of known virus sequences by

amplification using specific or degenerate primers; random amplification may be less useful, since all nonviral sequences would also be amplified. See Fig. 10.1.

Total nucleic acid recovery from samples complicates how sensitivity is defined and demonstrated. Such methods have the ability to recover nucleic acids from intact viral particles, cell genomes, and transcripts. Spike recovery experiments to establish sensitivity would arguably need to address each of these possibilities, though perhaps a worst case could be used in routine testing (for instance, an RNA virus spike since it would reflect release from viral particle as well as recovery of less stable RNA).

A common critique of total nucleic acid extractions is that they can recover nucleic acid remnants remaining after inactivation treatments of medium components, raw materials, and other reagents during sample preparation. Therefore, a complementary approach is extraction from intact viral particles. Intact viral particles with intact genomes represent potentially infectious agents. Extraction of nuclease-protected DNA and RNA recovers viral genomic nucleic acids, but could also recover some histone-protected cellular chromatin and/or free nucleic acids left by declining activity or below the affinity of the nuclease enzymes. These nuclease-resistant nucleic acids from cells, culture supernatants, or raw material fluids are well-suited to random-primed amplification steps.

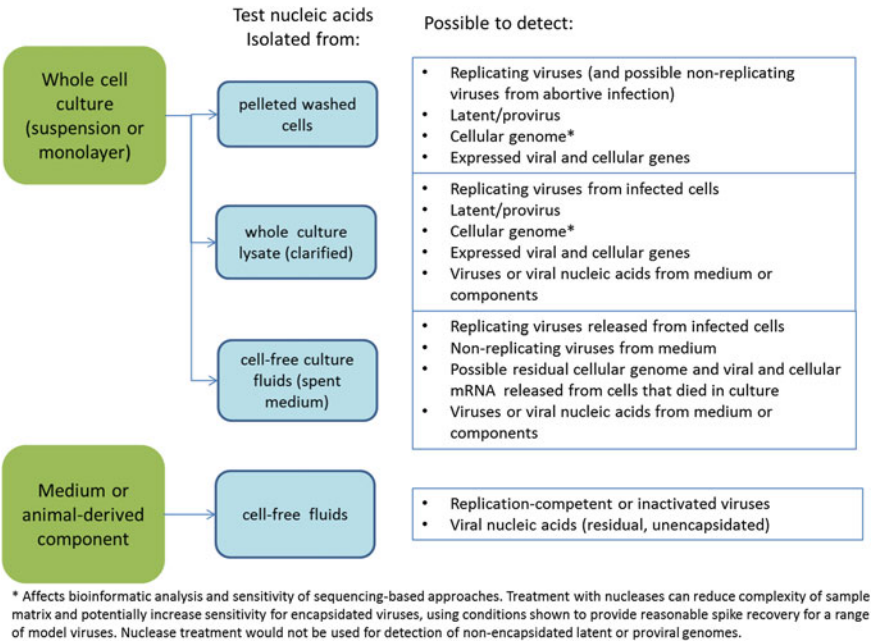


Fig. 10.1 Sample selection and preparation determine what can be detected

Additional treatments can be applied to concentrate potential viral particles as a means of enhancing sensitivity, such as ultracentrifugation or ultrafiltration. Where used, the efficacy of these methods for a range of viral particles needs to be demonstrated.

Database

The nucleic acid- and proteomic-based methods all rely on the arguably reasonable assumption that the next unknown virus will share at least some sequence similarities with known viruses for which sequences exist in the accessible databases. Coverage of sequences for a given virus, level of annotation, and curation vary in the public databases (EMBL in Europe, GenBank in USA, DDBJ in Japan). Curated reference genome databases (subsets of the public databases) provide reliable benchmarks, but do not necessarily represent the genetic diversity that might be available from partial sequences present in public databases. Private databases might be held within organizations that develop technologies, perform epidemiological surveys, or provide testing services. Such private databases might include data from public databases. Importantly, one must keep in mind that databases are not static because new sequences are added on an ongoing basis. One dilemma that the scientific and regulated testing community will need to address is whether or how to incorporate novel contigs assembled from next generation sequencing data, without viral isolation, into databases.

These critical characteristics of databases present a significant challenge not only to technology developers but also consumers of services based on information in the databases, and to regulators evaluating the data generated. The dynamic nature of databases makes PCR primer/probe design, array probe design, and any sequence searches subject to the version of database used at that point in time. Constant updating also presents a challenge for validation of assays and the frequency and timing of revalidation.

To the extent that private databases are generated and used for development of or application to adventitious agent detection technologies, there will be a need to scientifically validate them, and establish versioning and change control mechanisms for routine updates. Of course, not only must the databases be kept current, but methods based on them (for instance probes, PCR primers) must be re-evaluated to assure that they are current. When updates to databases render previous assays or searches out of date, users of the information may need to be informed of the update and the implications of changes. Users of these services might need to develop policies and procedures to determine when and how reanalysis is performed. Consequences of retesting could range from expenditures of time and resources investigating signals that turn out not to be true risks (and possible clinical or market actions in the meantime), to detection and identification of previously unrecognized infectious contaminations that tip the risk/benefit balance of the vaccine. Reanalysis of released materials goes against current quality assurance principles and so policy consideration needs to be given to this dilemma.

Sensitivity, False Positives, and False Negatives

Sensitivity viewed from the perspective of the readout alone might be described, for example, in copies per reaction for PCR- or hybridization-based assays. However, for biopharmaceutical applications, sensitivity needs to be interpreted in the context of the test article, which could be a volume of fluids or whole culture, or a pellet of cells from a culture or numbers of doses of the final product. Translating sensitivity back to the test article helps establish a bridge to the conventional methods. But determining what measure of sensitivity to apply for novel methods is not simple. Importantly, we must acknowledge that the readouts are not directly equivalent between conventional methods and novel, especially molecular, methods. Following are some issues that complicate equivalency arguments and influence how potentially positive and negative signals must be interpreted:

- not all viral genome copies are necessarily associated with viral particles, particularly in materials that have been subjected to inactivation or sterilization procedures
- not all viral particles contain viral genomes, although those that do not would not be infectious (but could complicate protein-based detection methods)
- not all viral particles with genomes are necessarily infectious
- some, but not all, viral genomes (if complete or largely so) in the absence of viral particles can be infectious or potentially oncogenic
- transcription of limited sets of viral genes does not necessarily reflect productive infection, although this signal might indicate an abortive infection, which could be of regulatory concern
- some reported viral sequences in public databases also contain nonviral sequences

It might appear that molecular methods are fraught with potential traps. However, this is not unique. Similar complications exist with conventional methods:

- infectivity will likely vary with cell substrate, and method of cultivation, and even method and route of inoculation in vivo due to varying tropism
- an infectious unit is not necessarily equivalent to a single intact viral particle with an intact genome
- viruses adapted to a cultivation system (for example, the manufacturing culture) could be easier to detect in cell culture-based infectivity assays than those present in an animal-derived raw material, which have not been exposed to in vitro cultivation
- infectivity in vitro might not reflect infectivity in vivo (either in assays, or as risk to the human receiving the medicine)
- infectivity may not equate to pathogenicity in the in vivo test systems or in human recipients of contaminated biologicals and may not equate to cytopathic effect in the tissue culture test systems used

Clearly, a complete and systematic validation of the sensitivity of conventional methods for all potential culture-adapted and wild viruses simply cannot be done.

Rather, judgments must be made for what is reasonable, practical, and represents scientific best practice to assure biosafety. For instance, the sensitivity of the novel methods can be characterized in their native units, such as genome copies, using appropriate spike controls as summarized in Fig. 10.2.

Another approach might compare earliest or lowest concentration detection between novel and conventional detection systems following infection of production or detection cells with panels of viruses with known infectious titers.

Thus, we argue that a reasonable starting point for establishing sensitivity of novel methods can be defined according to a few simple principles: See Fig. 10.2.

- If the novel method purports to detect viral nucleic acids from viral particles, then the effectiveness of the entire procedure must be demonstrated, starting with virus spikes representing both RNA and DNA genomes and various particle structures.
- If the novel method purports to detect proviral or latent viral DNA from preparations of total cellular DNA, then the effectiveness of the procedure for detecting levels of spiked viral or proviral nucleic acids must be demonstrated. Whether the spikes should be in the same form as the DNA intended to be detected (e.g., integrated into cellular DNA in the case of proviruses) needs consideration.

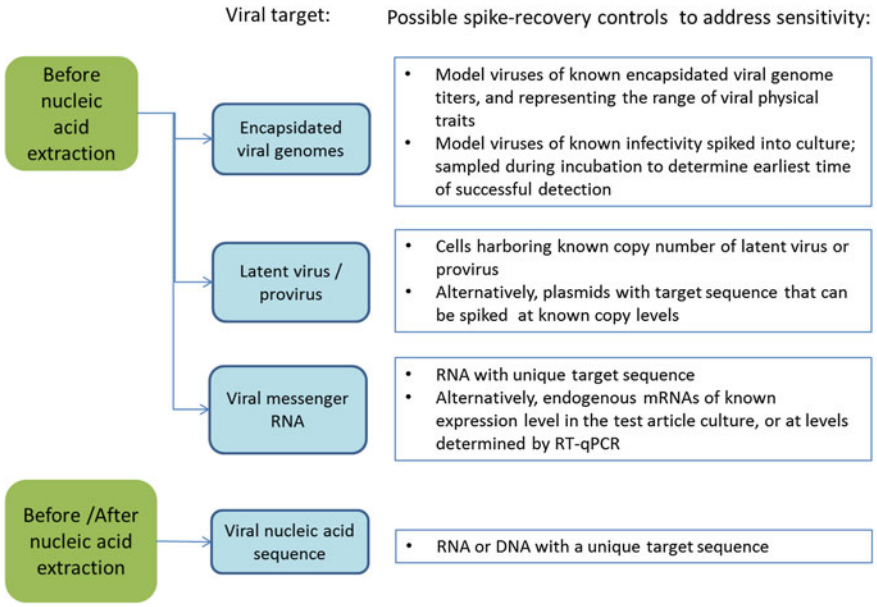


Fig. 10.2 Viral target determines the appropriate controls

- If the novel method purports to detect viral mRNA (transcripts), then the minimum detectable level of spiked RNA sequences must be demonstrated, bearing in mind potential bias that could be introduced by some mRNA purification methods.
- Detection of an absolute physical attribute of a virus (like a sequence) can be related to a relative measurement of potential infectious virus or virus risk by considering the totality of data—breadth and depth of sequence detected, identity, pattern of detection in the various controls (such as the corresponding medium or time-zero samples), and evidence of amplification when inoculated into uninfected cultures, for instance. Unfortunately, there is no simple relationship applicable to all viruses, or even all preparations of a single virus, that allows us to say that a certain amount of gene copies equates to a certain amount of infectious virus or virus risk. While one could argue that the worst case assumption is one gene copy per infectious unit, this is not the reality for most viruses.

Comparison of novel and conventional methods can perhaps be approached as follows or by variations on these:

- Spike relevant test article matrices with infectious virus standards and determine the lowest concentrations (i.e., highest dilutions) that can be detected in either production or detection assay cell systems. Virus stocks calibrated by both infectivity and genomic methods would be useful, as they help define comparability from batch to batch of the reference standard, and they help evaluate the molecular method in units relevant to the performance of those methods.
- Sample infected cultures through a timecourse, or use mixtures of infected and uninfected cultures resulting in differing ratios of infectious virus to background material, and determine the earliest timepoints and/or highest dilutions at which the adventitious agent is reliably detected in each method. This approach does not necessarily require calibration of the viral stock by both methods, although that calibration would still be useful.

Closely related to method sensitivity is the concept of a false negative result. Once the limit of detection for a method is defined, a false negative result is one for which a true contaminating agent was present at levels that should have been detectable, but went undetected. False negative results suggest a systematic error that prevents the detection of agents that could be present. Extra controls may be necessary for some time to establish reliability of all steps in the methods across changes in analysts, reagents, kits, test article lots, as well as database and software versions (the same could be accomplished through stringent change control procedures). Of course, the objective of these controls is to demonstrate lack of inhibition of spike recovery at or near the limit of detection of the method.

False positive signals can lead to unwarranted effort for follow-up investigations. There are at least three types of potential "false positives"—those due to lack of specificity, those due to contaminated reagents but unrelated to test article, and those that otherwise do not support a compelling assertion of presence of virus.

The last of these is considered further in Sect. "Evaluating Positive Signals." A failure of specificity can result in a signal being interpreted to represent a specific virus or virus sequence, when in fact it does not. Such signals can occur in the new detection systems by several mechanisms, and each of these needs to be controlled for the systems to be reliable. Targeted primers or probes may not be designed with necessary specificity, or reaction conditions that affect specific annealing might not be well controlled. There may be cellular analogs to some viral sequences. The accessions used in defining primers and probes, or evaluating sequence reads, may themselves be contaminated with cellular or vector sequences, especially if partial viral sequences or noncurated full genomes are used. There may also be considerable similarity among accessions in some sequences, which can result in signal for many accessions that do not represent the best identification. Microarray and PCR/Mass spec software incorporate complex algorithms to determine how much signal (depth and breadth, so to speak), is needed to assert a compelling positive detection. Such algorithms are yet in development for sequencing-based systems. Likely, as the ability to assemble short sequence reads into longer contigs improves, the confidence in positive hits and best hits will also improve. Reagents themselves can contribute signals due to residual nucleic acids from their preparation—notably even silica used in extraction kits (Smuts et al. 2014). But inevitably, some positive signals cannot be easily ascribed to errors, lack of specificity, or reagents. We differentiate these kinds of positive signals, and explore some principles for their further evaluation in Sect. "Evaluating Positive Signals" below.

Evaluating Positive Signals

Novel methods are susceptible to false positive signals in some interesting ways, beyond ordinary cross-contaminations. As a result, study design may be very important for interpretation of signals from molecular-based novel methods. Understanding the landscape of typical contaminants will help discriminate unusual ones. One means of doing this is by including various control samples including several negative controls. Understanding the manufacturing process for the vaccine and the various inputs is also critical to evaluating signals—for instance, where process interventions fully inactivate viruses but do not necessarily remove the viral sequences. Interpreting signals across multiple assays (for instance, total RNA as well as viral particle preparations) or even multiple samples can also help reveal viral amplification. For instance, an increase in viral signal with time in culture could strongly suggest productive infection. Positive results in transcriptome studies would be suggestive of infection and gene expression, even if not direct evidence of viral replication (especially in the case of latency transcripts). Mass balance estimates based on input and output copies can be useful for manufacturing cultures exposed to viral sequences and/or particles via raw materials of animal origin.

The observation of porcine circovirus sequences in a rotavirus vaccine by next generation sequencing and confirmation by microarray were strongly suggestive of infection, since there was considerable depth as well as complete breadth of viral

genome coverage. But these methods, as applied, did not completely prove presence of infectious virus in the vaccine. Proof was gained by evaluating predecessor materials and process inputs, and establishing assays for infectious porcine circovirus (http://www.fda.gov/AdvisoryCommittees/CommitteesMeetingMaterials/BloodVaccinesandOtherBiologics/VaccinesandRelatedBiologicalProductsAdvisoryCommittee/ucm197728.htm Accessed 13 Feb 2014). Recently, the World Health Organization has reviewed procedures by which regulatory agencies should evaluate positive signals in molecular (or any new) assay for adventitious viruses in biological products. Their recommendations have been adopted by their Expert Committee on Biological Standardization in Oct. 2014 and are anticipated to be published in 2015.

10.2.4.4 Implementing Novel Readouts

This section will address the manners in which novel readouts might be incorporated into quality control testing for adventitious agents, recognizing that many of these methods are already being used as part of viral risk assessment or in investigations. The options are relatively simple: readouts can be applied directly to samples of interest without opportunity for biological amplification in production cells or analytical indicator cells or animals, or they may be applied after these opportunities for biological amplification. The following table illustrates the potential applications of novel methods in relation to conventional methods, and illustrates the potentially broader utility of the novel methods (left side in Table 10.2). Importantly, the various readouts do not necessarily reflect the same biological properties, and thus relevance. Detection of nucleic acids may or may not reflect presence of an infectious virus, but only remnants of inactivated ones. Thus, the biological relevance of particular readouts is determined in large part by how and to what the novel detection method is applied.

We will refer to *direct tests* as those that are independently applied *without* a biological assay culture system, and *hybrid tests* as those *with* an initial biological amplification system. The suitability of any given readout, especially for direct testing, depends on sensitivity, sample preparation, and susceptibility to interference and false positives. For instance, immunofluorescence is not applied as a direct test but rather only after inoculation of cell cultures due to the need to biologically amplify and spatially concentrate signal for detection by microscopic examination. Conceptually, hemagglutination could be applied directly to test articles such as liquid raw materials without previous biological amplification in cells or animals, but to our knowledge is not, undoubtedly due to inherent low specificity, interference and/or insensitivity.

Adoption of a novel readout as a direct test to replace any conventional testing will likely require a very strong case for suitability (equivalent or better than existing methods, or at least arguably providing an acceptable safety margin where direct equivalency is not readily interpretable). Adoption of a novel readout as part of a hybrid assay (after biological amplification in analytical indicator cells or

Table 10.2 Sample and readout compatibility

The readouts below can be applied to samples from the right	Samples from		
	Raw material (i.e., media or components)	Production cultures (or corresponding control cell cultures)	Indicator cell cultures (whether applied to testing of raw materials or production or control cultures)
CPE	–	Adherent cells	
Proteins detection by:			
Hemadsorption	–	Adherent cells	
Hemagglutination	–	Fluids	
Specific immunofluorescence	–	–	Cells
Proteomic-mass spec	?	Fluids or cells	
Nucleic acids detection by:	+ (all)		
PCR/multiplex/ degenerate			
oligos/MS			
Microarray			
Next generation sequencing			

animals) will likely require a very strong business case to justify the added expense in addition to the scientific case for comparability (equivalent or better than existing methods).

Direct Testing with the Novel Readout

Direct testing of raw materials, media, manufacturing cultures, or fluids presents challenges of biological relevance and sensitivity. Detection of signal by most novel readouts does not necessarily prove presence of an infectious or relevant adventitious agent, but rather would be the starting point of an investigation into relevance of the signal. Given the high probability of detecting signal(s) of animal viruses in animal-derived raw materials even after they have been subjected to inactivation procedures, such as gamma irradiation, quality control, or release tests representing biological function might seem most appropriate. However, direct testing with novel readouts could increase the understanding of the landscape of potential agents to be considered as potential safety risks, and for which suitable assays for biological function might be needed. Few reports exist in the scientific literature of systematic surveys of animal-derived raw materials by the novel readouts for potential agents of concern, though it is likely that companies and technology developers are privately pursuing this objective.

Additionally, anything less than 100 % sampling and testing of a raw material is inevitably insufficient to assert that the material is absolutely free of detectable contaminants. Current testing paradigms rely on verification tests of animal-derived components coupled with validated processing of those components to reduce risk. It is also likely that improved sample preparation workflows will be needed to achieve meaningful levels of sensitivity for direct tests by the novel readouts. But most compelling is the increased breadth of detection that the novel readouts promise compared with the conventional tests.

Direct testing with the novel readouts could also be used to support in-process decision-making or to replace conventional tests on production cultures and harvests. The wider breadth of detection would arguably provide greater assurance that unexpected agents have not propagated in any given manufacturing culture. Appropriate controls, such as uninoculated complete medium or a "time zero" culture sample, would be essential to support that detected signals actually show an increase during the culture step (and should have been shown previously to be undetectable in the cell substrate). We address additional nuances of sensitivity of such tests in Sect. 10.3, in the context of the viral safety margin.

Hybrid Test Using Novel Readout

Novel readouts could be effectively applied in hybrid assays, just like conventional readouts. As used here, hybrid assays are those in which the test article has been exposed to a conventional or nonconventional analytical cell or animal system in which biological amplification of the agent could occur, and a separate nonconventional readout is applied to detect the agent. A conventional cell culture-based assay might be validated to detect "100" infectious units of a virus (whether defined as TCID50 or PFU). But the readouts typically do not actually detect the initial 100 units, but rather the effects of virus growth started with those 100 infectious units. The biological test system facilitates amplification of the virus inoculum, initially undetectable in the conventional readout, to levels of virus or impact on infected cells that are readily detected by the conventional readout. The same would be true of a novel readout. In fact, detection of the increase in genome copies of porcine circovirus 1, which did not generate CPE, was key to its detection in an infectivity assay (presentation by Krause, http://www.fda.gov/AdvisoryCommittees/CommitteesMeetingMaterials/BloodVaccinesandOtherBiologics/VaccinesandRelatedBiologicalProductsAdvisoryCommittee/ucm211828.htm Accessed 13 Feb 2014). Hybrid approaches have also been proposed for mycoplasma testing (Chang et al. 2006; Kong et al. 2007). Thus, novel readouts could be applied to analytical cultures to enhance detectability of viruses and other organisms.

One consideration that may be given to the validation of the hybrid test is that only the novel readout would require validation, as the biological amplification would occur as it is already performed by the compendial methods (which are not validated, and arguably cannot truly be validated). This would not necessarily be

the case for a nonstandard biological amplification method, but should be a suitable approach for the compendial methods.

The challenge with hybrid assays is that they incur the logistics and cost of both the biological amplification step and the molecular readout. It is perhaps most logical to develop a hybrid test approach only once it is concluded that the direct test approach is inadequate.

In the next section, we consider a rational strategy based on assessment and selection of inputs, design of manufacturing processes, and testing. These steps illuminate how test sensitivity fits into the overarching viral safety margin, and thus how it supports the scientific and business case for selecting a novel testing strategy.

10.3 Principles of Rational and Scientifically Based Testing Strategies

Viral safety assurance is best described as a confidence-building exercise with multiple contributing factors rather than as a quantitative measure of any one component. Nonetheless, selecting testing strategies to assure viral safety inherently requires that choices be made about what to test for (breadth) and how sensitive the tests should be (combination of inherent method sensitivity, any potential biological amplification, and sample preparation and size). Viral risk assessments, process knowledge (including inputs), and testing methods each inform, and are informed by, the others, resulting in a critical triad on which viral safety is established. (See Fig. 10.3).

A rational strategy to assure biosafety then incorporates these three elements, risk assessment, process knowledge (including inputs), and the analytical methods used to detect potential agents. In the following section, we discuss risk assessment and process choices as a means of building context around the viral safety margin, and just how the analytics contribute. We conclude by reflecting on the regulatory and business cases for adopting new analytical approaches.

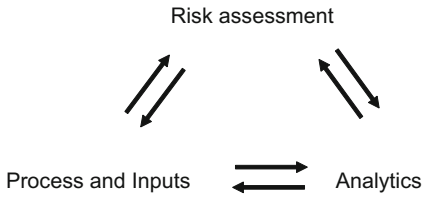


Fig. 10.3 Contributions to viral safety assurance

10.3.1 Elements of Risk Assessment and How Process Choices Mitigate Viral Safety Risk

Although there is a tendency to want to take a checklist approach to testing for biosafety in vaccines, and compendia and regulations support this inclination, such an approach is neither suitable nor scientifically supportable. Which agents may be adventitious in a particular vaccine production system or platform technology depends entirely on a large number of factors. These factors must be considered rationally in deciding testing strategies. In addition, particularly in an era of Quality by Design (QbD), an assessment of risks should be undertaken to guide this decision-making process. Factors that should weigh into the considerations of testing strategies include, but are not limited to, those found in Table 10.3.

In addition to the factors in Table 10.3, the risk assessment process needs to take into account the likelihood of an agent to be present, the likelihood of its detection, if present, and the severity of the impact, if not detected. Not only is the likelihood of the presence or absence of a specific agent to be considered, but also the impact of its presence is considered during risk assessment. Highly pathogenic viruses, even if unlikely to be present, could be of higher concern than nonpathogenic agents, even if likely to be present. Furthermore, the detectability of a particular contaminant must also be considered, with agents unlikely to be readily detected potentially being of higher concern than those that are readily detectable. This aspect is discussed further in the following section.

10.3.2 Testing in the Context of Different Risk Scenarios

We have already alluded to the gap in breadth of detection of conventional methods. Thus, agents could be present in cultures that were not expected and for which tests were not designed. The resolution to this gap is to incorporate assays with broader detection capability, either to replace or supplement existing methods.

We have also reflected on the existing expectations for sensitivity, and the relative lack of an enduring, well-described rationale on acceptable safety margin for viral vaccines. By viral safety margin, we mean the excess capacity of a process for assuring any potential contaminant is removed, inactivated, or simply not present beyond what was present originally (if any). Considering viral clearance validation as an example, if an endogenous viral contaminant is present at such a level that virtually every dose of vaccine produced could contain particles, then viral clearance would be necessary to clear the virus particles to a level that establishes that statistically only a vanishingly small number of doses could potentially be contaminated in a whole lot. Often the regulatory expectation is that this margin-of-safety reduces the probability to less than 1 in a million doses being likely to contain a particle, for instance, in the illustration provided in ICH Q5A (ICH 1999). This excess capacity of a process to clear viruses that might be present

Table 10.3 Factors relevant to choosing a viral safety testing strategy

In regards to	Risk identification
Cell substrate origin	From which species and tissue is the production cell substrate derived?
	What was the health status of the cell substrate donor(s)?
Legacy of cell substrate	What has happened in the legacy of the cell substrate derivation and establishment, including which species and geographic region provided the raw materials to which the cell substrate has been exposed and what were those materials?
Banking of cell substrate	Is the cell substrate banked or primary?
Origin and legacy of vaccine virus	From where (geographically) and from which species was the vaccine virus isolated originally (before adaptation to develop vaccine strain)?
	To which species or cell substrates (and were they well-characterized) has the viral isolate been exposed in adapting it to become a vaccine strain?
	Has the vaccine strain been passaged through alternative species to provide a species barrier to adventitious viruses that may have been present?
	From which species are the raw materials, including antisera that may have been used for selection and adaptation purposes, to which the viral isolate or vaccine strain has been exposed during derivation or adaptation?
Cloning of vaccine virus	Has the vaccine virus been molecularly cloned or biologically cloned by limiting dilution and if the latter, how many times (i.e., is it even numerically or physically possible that an adventitious agent could still be present from legacy)?
Handling of vaccine virus before seed was banked and during banking	To what materials, including cell substrate(s), has the vaccine virus been exposed since cloning, if applicable?
	In what environment (field surveillance laboratory, basic science laboratory, industrial R&D laboratory, GMP suite) has the vaccine virus been handled prior to and during the production of the master viral seed and/or working viral seed (from isolation to preproduction of vaccine)?
	What other viruses and cell substrates have been handled in the same environment(s) in the timeframes prior to or during this handling?
Facility for vaccine production	How are the environment and personnel controlled and monitored during vaccine production (health status, personal protective gear, training in aseptic processes, SOPs in place to prevent and control contamination and cross-contamination, personnel and materials flow, positive or negative pressure, room classification, environmental and personnel monitoring)?
	What is the state of validation of equipment, including HVAC, and production facilities used during vaccine production?
Vaccine production	What are the production processes and are the processing steps aseptic or controlled for bioburden?
	Are there viral inactivation or clearance steps incorporated into the production processes?
	From which species and geographic region are raw materials used during production derived?
Quality control	What tests and detection technologies are available for quality control and for characterization?
Drug product filling	How is filling of product final containers controlled?
Clinical use	What is the pathogenicity or capacity to infect human recipients of vaccines, as well as the susceptibility of the target population (e.g., children) for the vaccine in question, of specific potential contaminants?

(e.g., of residual live virus in the case of inactivated viral vaccines or of endogenous viral particles in the case of recombinant vaccines made in rodent cells) is the viral safety margin. Viral clearance validation or validation of inactivation procedures for inactivated vaccines are intended to establish such viral safety margins. We find this concept useful in thinking about the needed sensitivity of viral detection methods to ensure freedom from adventitious agents and consider whether conventional or novel methods can attain such, as discussed below.

Models can help build some of the necessary quantitative context, and would take into account the load from materials and even previous steps, ability to replicate in the manufacturing system, ability to be cleared or inactivated, and proportion of the culture used to make a final dose. Output of a model could include doses of vaccine per infectious unit of the potential virus with specified growth characteristics. An intermediate value in the calculation, infectious units/mL of culture, could be compared with the known sensitivities of the conventional detection method as well as the novel method, allowing some visibility into whether the limits of detection or breadth of detection of either analytical method really provides a meaningful viral safety limit compared with that contributed by the manufacturing process and controls. The challenges with such a modeling approach become obvious—many values are required for which solid experimental data may not be available. Clearly, recalibration would be necessary, requiring another assumption, to convert from infectious units to viral genome copy equivalents to enable these comparisons. On the other hand, simply wrestling with these gaps in knowledge could lead to hypotheses for experiments or alternative approaches to support a more scientific rationale for the viral safety margin, as well as inform the risk assessment, thus applying the principles of QbD. Others have offered approaches to viral risk assessments that incorporate some of these features (Gregerson 2008a, b; Tagmyer 2012), although they do not focus on the quantitative evaluation of alternative detection methods or suggest which methods might be best to use as an outcome of the assessment process.

Some quantitative aspects of such a model, e.g., on the detectability of an adventitious agent introduced by a raw material during production at the stage of cell culture would require actual data or valid assumptions. These would include, but not necessarily be limited to: the volume of the raw material and the culture to which it is added, concentration (titer) of the agent (if nothing detected by testing the bulk raw material, then assume LOD of detection method), log reduction value (LRV) based on validation data of any inactivation method applied to the raw material before introducing it into the cell culture, ability to replicate in the culture and burst size or amplification factor, length of culture period (how many replication cycles could occur) and length of replication cycle of agent, LRV afforded by any handling/storage of specimens taken for adventitious agent testing of the culture or downstream processing before specimen collection occurs, volume of the culture that results in a dose of vaccine, the LOD of the detection methods (conventional and novel), viral safety margin one is trying to achieve, and so forth.

We are confident that mathematical models will demonstrate that testing alone does not establish the viral safety margin, and a broader range of detectability is far

more important in assuring absence of a catastrophic breach in the manufacturing process than rigidly adhering to arbitrary and nonstandardized approaches to sensitivity, based on conventional tests. This is the only way that a rational and scientifically based strategy can emerge, and not result in simply adding each new test or technology to an ever-increasing list of tests.

It is problematic to continue adding to the ever-growing list of tests for a number of reasons. The amount of sample needed for QC testing and reserve specimens would be ever increasing and often, more complicated, as different tests need differing material (e.g., cells, fluids) to be sampled, as well as processed, handled, and stored differently. It will become fiscally unsustainable when manufacturing needs to be scaled-up simply to account for QC samples (as is sometimes the case for Phase 1 clinical lots), in addition to the costs of developing, validating, and conducting all of the required testing. The more tests that are run, the more likely that a false positive result will be observed, if each test is validated to a confidence level of 95 % (i.e., then statistically 5 % of tests could be falsely positive by chance). And finally, and importantly, discordant results on tests that measure the same parameter (i.e., detection of viral contaminants) could become a QA/QC quagmire. Manufacturers would need to prospectively determine an algorithm not only for validity criteria within a single test, but also for which test to "believe" or what testing strategy would need to be undertaken to resolve the matter in the case of discordant results between tests for the same measure. And regulators, as well as the public, could lose confidence if they see such discordant results, even if carefully investigated and decided upon with such prospectively identified algorithms by the manufacturer. Thus, we propose that a new paradigm is needed, based on a broader platform for viral safety assurance that incorporates viral risk assessment, process and input controls, as well as improved analytics.

10.3.3 Making the Regulatory and Business Case for New Analytical Approaches

Both the regulatory and business cases for adopting novel methods is arguably based on the perceived gap in safety assurance. If the gap is perceived as large, then the investment in resources for developing, validating, implementing, and running novel methods is relatively easy to defend.

Currently a gap in viral coverage of existing testing is recognized, particularly in the case of novel cell substrates and biologically derived raw materials. Emerging methods have the potential to not only close the gap for unexpected viruses, but also arguably to provide coverage for the expected viruses. The challenge for manufacturers, contract testing laboratories, and regulatory bodies will be to assess the credibility of negative results—that is, how much assurance of lack of contamination do they really provide—since positive results presumably will be evaluated with orthogonal confirmatory approaches. Studies designed to carefully

interpret negative results necessarily include sets of controls that increase the cost, and sometimes the complexity of sampling, and most assuredly the complexity of data interpretation and establishment of specifications. Specifications themselves may have to allow certain levels of signal that can be interpreted as noninformative or noncompelling as an indicator of contamination with a certain accepted level of confidence.

Biopharmaceutical manufacturers that use contract services for novel methods face the challenge of potentially not having in-house technical expertise on the methods, while still owning liability for oversight or incomplete or incorrect analysis. Vaccine developers also incur risk to development program timelines while investigating false positive results, risk of unwarranted comfort in the face of false negative results, and perhaps uneven regulatory expectations (e.g., if one regulatory region embraced particular new methods more readily or more rapidly than others, as has been seen with PCR for mycoplasma detection). Importantly, as both the technology landscape and perceived risks evolve over time, biopharmaceutical manufacturers may be faced with the significant "change control" issues—for instance that might be presented by updated databases or improved detection limits. The business risks for biopharmaceutical projects in early development are also quantitatively different than those for legacy products with strong safety records (and which often supply essential public health needs, sometimes as sole sources).

Businesses that develop and provide these services face the challenges of a rapidly evolving technology landscape, acceptance/understanding by customers and regulators, and sustaining focus over timescales relevant to biopharmaceutical development and licensure, and undoubtedly intense cost pressure.

Businesses and regulatory agencies face a shared dilemma when incorporating novel approaches for legacy products. Neither the methods nor the landscape of potential signals are fully understood yet. Prudent review of method development, surveys of materials, and robust courses of action for investigating potential positive hits in NAT-based tests will be needed to avoid potential risk that the public might lose access to or confidence in critical disease-preventing or disease-treating vaccines.

10.4 Summary

In summary, this chapter reviews the principles of how the current and routine tests detect adventitious agents, and reviews how novel and emerging methods differ in their detection principles. These facets may permit novel methods to emerge to supplement, refine, or replace the routine methods. We have suggested a framework for risk assessment to assure biosafety in vaccines and suggested quantitative modeling to help crystallize thinking about the place of testing, either routine or novel, in this assurance. We assert that testing for adventitious agents should not be the sole basis on which product biosafety is assured. Appropriate sourcing and

quality control of raw and starting materials, adherence to principles of Good Manufacturing Practices, including environmental and personnel monitoring and process validation, and finally, testing as verification are the package needed for maximal assurance of biosafety. Thus, a pathway forward to a new paradigm for adventitious agent testing exists in which detection of a broader array of potential adventitious agents might be included in the testing, with adequate sensitivity to provide the needed assurance of verification that there has been no catastrophic breach, in the context of the overall process, design, and adherence to cGMP. Furthermore, it is our hope that we may be able to implement the 3 Rs policy to reduce, replace, and/or refine the use of animals in product safety testing, at the same time that we provide greater assurance of the biosafety of vaccines.

Acknowledgments The authors wish to acknowledge those who assisted in the crystallization of ideas for this chapter. For intensive statistical discussions about margin-of-safety, from the National Institutes of Health's Biostatistics Research Branch, Drs. Martha Nason and Michael Proschan. We would also like to acknowledge helpful discussions with Robert Capen, Tara Tagmyer, Szi-Fei Feng, Chris Wang and Khamir Mehta at Merck.

References

Adler M, Schulz S, Fischer R, Niemeyer CM (2005) Detection of Rotavirus from stool samples using a standardized immuno-PCR ("Imperacer") method with end-point and real-time detection. Biochem Biophys Res Com 333:1289–1294

Berthet N, Leclercq I, Dublineau A, Shigematsu S, Burguière AM, Filippone C, Gessain A, Manuguerra J-C (2010) High-density resequencing DNA microarrays in public health emergencies. Nat Biotechnol 28:25–27

Boni J, Pyra H, Schupbach J (1996) Sensitive detection and quantification of particle-associated reverse transcriptase in plasma of HIV-1-infectived individuals by the product-enhanced reverse transcriptase (PERT) assay. J Med Virol 49:23–28

Callendo AM (2011) Multiplex PCR and emerging technologies for the detection of respiratory pathogens. Clin Infect Dis 52(Suppl 4):S326–S330

Chang A, Armstrong A, Prud'homme I, Hsieh W (2006) Using quantitative polymerase chain reaction (Q-PCR) for mycoplasma testing of biopharmaceutical products: the HyMy™ assay. In: Miller M (ed) Encyclopedia of rapid microbiological methods, vol III, Chap. 14. PDA/DHI, Bethesda/River Grove, pp 309–316

Chen KF, Blyn L, Rothman RE, Ramachandran P, Valsamakis A, Ecker D, Sampath R, Gaydos CA (2011a) Reverse transcription polymerase chain reaction and electrospray ionization mass spectrometry for identifying acute viral upper respiratory tract infections. Diagn Microbiol Infect Dis 69(2):179–186

Chen KF, Rothman RE, Ramachandran P, Blyn L, Sampath R, Ecker DJ, Valsamakis A, Gaydos CA (2011) Rapid identification viruses from nasal pharyngeal aspirates in acute viral respiratory infections by RT-PCR and electrospray ionization mass spectrometry. J Virol Methods 173(1):60–66 (Epub 2011 Jan 21)

Chou T-C, Hsu W, Wang C-H, Chen Y-J, Fang J-M (2011) Rapid and specific influenza virus detection by functionalized magnetic nanoparticles and mass spectrometry. J Nanobiotech 9:52

Code of Federal Regulations Title 21 Parts 630.18(a) and 630.60(c) (1996) Poliovirus vaccine live oral trivalent additional tests for safety and Rubella virus vaccine live. Government Printing Office, Washington DC

Code of Federal Regulations Title 21 Part 610.9 (2012a) Equivalent methods and processes. Government Printing Office, Washington DC

Code of Federal Regulations Title 21 Part 11 (2012b) Electronic records; electronic signatures. Government Printing Office, Washington DC

Cornfield J, Halperin M, Moore F (1956) Some statistical aspects of safety testing of the salk poliomyelitis vaccine. Public Health Rep 71(10):1045–1056

de Vries M, Deijs M, Canuti M, van Schaik BD, Faria NR, van de Garde MD, Jachimowski LC, Jebbink MF, Jakobs M, Luyf AC, Coenjaerts FE, Claas EC, Molenkamp R, Koekkoek SM, Lammens C, Leus F, Goossens H, Ieven M, Baas F, van der Hoek L (2011) A sensitive assay for virus discovery in respiratory clinical samples. PLoS One 6(1):e16118

Deyde VM, Sampath R, Gubareva LV (2011) RT-PCR/electrospray ionization mass spectrometry approach in detection and characterization of influenza viruses. Expert Rev Mol Diagn 11 (1):41–52

Donald HB, Isaacs A (1954) Counts on influenza particles. J Gen Microbiol 10:457–464. http://mic.sgmjournals.org/content/10/3/457.long. Accessed 13 Feb 2014

Ecker DJ, Sampath R, Li H, Massire C, Matthews HE, Toleno D, Hall TA, Blyn LB, Eshoo MW, Ranken R, Hofstadler SA, Tang YW (2010) New technology for rapid molecular diagnosis of bloodstream infections. Expert Rev Mol Diagn 10(4):399–415

Erlandsson L, Rosenstierne MW, McLoughlin K, Jaing C, Fomsgaard A (2011) The microbial detection array combined with random Phi29-amplification used as a diagnostic tool for virus detection in clinical samples. PLoS ONE 6(8):e22631. doi:10.1371/journal.pone.0022631

European Medicines Agency (EMA) (2011) Note for guidance on minimising the risk of transmitting animal spongiform encephalopathy via human and veterinary medicinal products (EMA/410/01 rev. 3). http://www.emea.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003700.pdf. Accessed 19 Dec 2012

European Pharmacopeia Version 8.0 (2014a) Monograph 2.6.16 (01/2011:20616) tests for extraneous agents in viral vaccines for human use. EP8.0:198–200

European Pharmacopeia Version 8.0 (2014b) Monograph 5.2.3 (01/2011:50203) cell substrates for the production of vaccines for human use. EP8.0:582–585

FDA/CBER/OVRR (2012a) Recommendations for the use of vaccines manufactured with bovine-derived materials. http://www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/ucm111476.htm. Accessed 19 Dec 2012

FDA/CBER/OVRR (2010) Guidance for industry: characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines for infectious disease indications. http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/UCM202439.pdf. Accessed 19 Dec 2012

FDA/CBER/OVRR (2012b) 2012 meeting materials, vaccines and related biological products advisory committee. http://www.fda.gov/AdvisoryCommittees/CommitteesMeetingMaterials/BloodVaccinesandOtherBiologics/VaccinesandRelatedBiologicalProductsAdvisoryCommittee/ucm288695.htm. Accessed 19 Dec 2012

Glenn T (2011) Field guide to next generation DNA sequencers. Mol Ecol Resour 11:759–769

Gombold J, Karakasidis S, Niksa P, Podczasy J, Neumann K, Richardson J, Sane N, Johnson-Leva R, Randolph V, Sadoff J, Minor P, Schmidt A, Duncan P, Sheets RL (2014) Systematic evaluation of in vitro and in vivo adventitious virus assays for the detection of viral contamination of cell banks and biological products. Vaccine (in press)

Gregersen J-P (2008a) A risk-assessment model to rate the occurrence and relevance of adventitious agents in the production of influenza vaccines. Vaccine 26:3297–3304

Gregersen J-P (2008b) A quantitative risk assessment of exposure to adventitious agents in a cell-culture-derived subunit influenza vaccine. Vaccine 26:3332–3340

Hall A, Koehler J, Rolfe A, Minogue T (2012) Use of selective sequencing probes for pathogen identification by next-generation sequencing. In: 7th annual sequencing, finishing and analysis in the future meeting, Department of Energy Office of Science and Joint Genome Institute, and

Los Alamos National Laboratory, Santa Fe New Mexico, 05–07 June 2012, p 75. http://www.lanl.gov/conferences/finishfuture/pdfs/guides/2012_meeting_guide.pdf. Accessed 19 Dec 2012

International Conference on Harmonisation (1996) Validation of analytical procedures: text and methods [Q2A(R1)]. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf. Accessed 13 Feb 2014

International Conference on Harmonisation (1999) Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin [Q5A(R1)]. http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html. Accessed 19 Dec 2012

Japanese Pharmacopeia (2011) General information 3: basic requirements for viral safety of biotechnological/biological products listed in the Japanese pharmacopeia. JP XVI:2166–2179

Karlsson R, Davidson M, Svensson-Stadler L, Karlsson A, Olesen K, Carlsohn E, Moore ERB (2012) Strain-level typing and identification of bacteria using mass spectrometry-based proteomics. J Proteome Res 11(5):2710–2720

Konietzny R, Fischer R, Ternette N, Wright CA, Turney BW, Chakera A, Hughes D, Kessler BM, Pugh CW (2012) Detection of BK virus in urine from renal transplant subjects by mass spectrometry. Clin Proteomics 9:4

Kong H, Volokhov DV, George J, Ikonomi P, Chandler D, Anderson C, Chizhikov V (2007) Application of cell culture enrichment for improving the sensitivity of mycoplasma detection methods based on nucleic acid amplification technology (NAT). Appl Microbiol Biotechnol 77 (1):223–232 (epub 2007 Aug 24)

König C, Engemann C, Krummrei U, Wacker R, Hoffmann R (2006) Detection of prions in body fluids—diagnosis of CJD. In: Poster presented at Prion 2006: Torino, Italy, 4–6 Oct 2006

Kozal M, Sheh A, Adesokan A (2012) Sequence based multiplex detection of NS mutations in HCV1a and 1b genotypes. In: Poster presented at international workshop on HIV & Hepatitis virus drug resistance and curative strategies, in Sitges, Spain, June 2012

Lawrence B, Bashiri H, Delghani H (2010) Cross-comparison of rapid mycoplasma detection platforms. Biologicals 38(2):218–223

Leland DS, Ginocchio CC (2007) Role of cell culture for virus detection in the age of technology. Clin Microbiol Rev 20(1):49–78

Leski TA, Malanoski AP, Gregory MJ, Lin B, Stenger DA (2011) Application of a broad-range resequencing array for detection of pathogens in desert dust samples from Kuwait and Iraq. Appl Environ Microbiol 77(13):4285–4292

Li JB, Gao Y, Aach J et al (2009) Multiplex padlock targeted sequencing reveals human hypermutable CpG variations. Genome Res 19:1606–1615

Lun ATL, Wong JWH, Downard KM (2012) Rapid identification of reassorted strains of pandemic influenza with mass spectrometry—May 21, 2012. In: ASMS Meeting 2012, Vancouver, BC, Canada

McClenahan SD, Uhlenhaut C, Krause PR (2014) Optimization of virus detection in cells using massively parallel sequencing. Biologicals 42(1):34–41

Metzker M (2010) Sequencing technologies—the next generation. Nat Rev Genet 11:31–46

Munroe D (2011) A microarray platform for virus detection and identification. PDA J Pharm Sci Technol 65(6):691

Nanda S, Jayan G, Voulgaropoulou F, Sierra-Honigmann AM, Uhlenhaut C, McWatters BJ et al (2008) Universal virus detection by degenerate-oligonucleotide primed polymerase chain reaction of purified viral nucleic acids. J Virol Methods 152:18–24

Niedringhaus TP, Milanova D, Kerby MB, Snyder MP, Barron AE (2011) Landscape of next-generation sequencing technologies. Anal Chem 83:4327–4341

Olivo PD (1996) Transgenic cell lines for detection of animal viruses. Clin Microbiol Rev 9:321–334

Petricciani J, Sheets R, Stacey G, Knezevic I, WHO Study Group on Cell Substrates (2010) Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks. WHO Tech Rep Ser 978, Annex 3:79–187. http://www.who.int/biologicals/vaccines/TRS_978_Annex_3.pdf?ua=1. Accessed 13 Feb 2014

Piccardo P, Cervenakova L, Vasilyeva I et al (2011) Candidate cell substrates, vaccine production, and transmissible spongiform encephalopathies. Emerg Infect Dis 17(12):2262–2269. doi:10.3201/eid1712.110607

Porreca GJ, Zhang K, Li JB, Xie B, Austin D, Vassall SL, LeProust EM, Peck BJ, Emig CJ, Dahl F, Gao Y, Church GM, Shendure J (2007) Multiplex amplification of large sets of human exons. Nat Methods 4:931–936

Roingeard P (2008) Viral detection by electron microscopy: past, present and future. Biol Cell 100:491–501. doi:10.1042/BC20070173

Sampath R, Blyn LB, Ecker DJ (2010) Rapid molecular assays for microbial contaminant monitoring in the bioprocess industry. PDA J Pharm Sci Technol 64(5):458–464

Sampath R, Mulholland N, Blyn LB, Massire C, Whitehouse CA et al (2012) Comprehensive biothreat cluster identification by PCR/electrospray-ionization mass spectrometry. PLoS ONE 7(6):e36528. doi:10.1371/journal.pone.0036528

Sandstron PA, Folks TM (2001) Facilitated detection of adventitious agents using genetically engineered cell lines. Dev Biol (Basel) 106:375–378

Schwahn AB, Wong JWH, Downard KM (2009) Subtyping of the Influenza virus by high resolution mass spectrometry. Anal Chem 81:3500–3506

Shelokov A, Vogel JE, Chi L (1958) Hemadsorption (adsorption-hemagglutination) test for viral agents in tissue culture with special reference to influenza. Exp Biol Med 97(4):802–809. doi:10.3181/00379727-97-23884

Sheng L, Cai F, Zhu Y, Pal A, Atthanasiou M, Orrison B, Blair DG, Hughes SH, Coffin JM, Lewis AM, Peden K (2008) Oncogenicity of DNA in vivo: tumor induction with expression plasmids for activated H-ras and c-myc. Biologicals 36(3):184–197

Sheng-Fowler L, Cai F, Fu H et al (2010) Tumors induced in mice by direct inoculation of plasmid DNA expressing both activated H-*ras* and c-*myc*. Int J Biol Sci 6(2):151–162

Smuts H, Kew M, Khan A, Korsman S (2014) Novel hybrid Parvovirus-like virus, NIH-CQV/PHV, contaminants in silica column-based nucleic acid extraction kits. J Virol 88(2):1398

Tagmyer T (2012) Assessing viral risk in anmial-derived raw materials used in vaccine manufacturing. Presented at PDA/FDA virus and TSE safety conference, parenteral drug association and us food and drug administration. 15–17 May, 2012, Bethesda, MD

Uhlenhaut C, Cohen JI, Fedorko D, Nanda S, Krause PR (2009) Use of a universal virus detection assay to identify human metapneumovirus in a hematopoietic stem cell transplant recipient with pneumonia of unknown origin. J Clin Virol 44:337–339

Victoria JG, Wang C, Jones MS, Jaing C, McLoughlin K, Gardner S, Delwart EL (2010) Viral nucleic acids in live-attenuated vaccines: detection of minority variants and an adventitious virus. J Virol 84(12):6033–6040

Wieser A, Schneider L, Jung J, Schubert S (2012) MALDI-TOF MS in microbiological diagnostics-identification of microorganisms and beyond (mini review). Appl Microbiol Biotechnol 93(3):965–974

Wong JWH, Schwahn AB, Downard KM (2010) FluTyper—an algorithm for automated typing and subtyping of the Influenza virus from high resolution mass spectral data. BMC Bioinformatics 11:266

Ye Y, Mar E-C, Tong S, Sammons S, Fang S, Anderson LJ, Wang D (2010) Application of proteomics methods for pathogen discovery. J Virol Methods 163(1):87–95

Zheng J, Sugrue RJ, Tang K (2011) Mass spectrometry based proteomic studies on viruses and hosts–A review. Anal Chim Acta 702(2):149–159

Zhou ST, Liu R, Zhao X, Huang CH, Wei YQ (2011) Viral proteomics: the emerging cutting-edge of virus research. Sci China Life Sci 54(6):502–512

Chapter 11
Deep Sequencing Applications for Vaccine Development and Safety

David Onions, Colette Côté, Brad Love and John Kolman

11.1 Introduction

The introduction of deep sequencing or massively parallel sequencing (MPS) technologies has resulted in a qualitative, as well as a quantitative, change in the information that can be gained from nucleic acid sequencing. It has revolutionised virus discovery by revealing new species and new genera of viruses that had gone undetected by conventional means. While the sequencing technologies are elegant, the key to their application is in the development of robust, automated, bioinformatics; the combination of sequencing and bioinformatic interpretation being termed MP-Seq. In the field of vaccine safety, another level of complexity is validating the many individual steps to ensure that the overall process is in compliance with Good Manufacturing Practice (GMP).

The underpinning principle of MPS is that many hundreds of thousands of strands within a nucleic acid population are sequenced without bias. These might be individual viral genome sequences or, mRNA transcripts in a transcriptome (RNA-seq) analysis. Consequently, the inherent variation in the population is recorded rather than a consensus sequence obtained by traditional sequencing methods. At the same time, the burden of identifying sequences of interest shifts. While DNA cloning or sequence specific PCR amplification defines the target for traditional sequencing, in an MPS analysis the targets of interest are extracted using bioinformatics.

MPS is used today as an adjunct to regulated nucleic acid based biosafety tests. It can be applied directly to virus seed stocks to establish virus genome identity and genetic stability. More challenging is the use of MPS to support vaccine development by evaluating cell substrates, raw materials and virus seed stocks for adventitious agents. Contamination of vaccines by adventitious viruses has a long

D. Onions (✉) · C. Côté · B. Love · J. Kolman
BioReliance, Sigma-Aldrich, 14920 Broschart Road, Rockville, MD 20850-3349, USA
e-mail: david.onions@hotmail.com; david.onions@bioreliance.com

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6_11

history and includes the finding of SV40 in early poliovirus vaccines, avian leukosis (leukemia) virus in yellow fever virus grown in eggs, bovine viral diarrhoea virus in veterinary vaccines and the feline retrovirus, RD114, in canine parvovirus vaccines.

As a result of early contamination events, a rigorous set of overlapping, specific and broad based, detection methods for adventitious agents was introduced to enhance biosafety safety. These include in vivo assays in eggs and animals and in vitro infectivity assays. Since the early 1990s, polymerase chain reaction (PCR) assays for specific viruses or families of viruses have been incorporated into the testing regime of vaccine seeds and cell substrates. In the latter case, the assays may be linked to induction protocols to enhance the expression of latent or endogenous viruses (Khan et al. 2009; Onions et al. 2010).

Despite the breadth of these assay systems, new contaminations occur, either because the virus was unknown or, because specific testing was not incorporated. One of the most recent examples was the finding of porcine circovirus sequences in rotavirus vaccines using MPS (Victoria et al. 2010). In one vaccine, the sequences were shown to be associated with infectious circovirus indicating a failure of traditional testing strategies to detect the virus. Two lessons should be drawn from this recent example. It illustrates the power of MPS to detect agents without any assumptions of the nature of the agent. Secondly, technology on its own, is not a solution, testing needs to be incorporated into a holistic, quality by design (QbD) approach that evaluates the potential risks of each element of production including raw materials, the cell substrate and virus seed.

11.2 New Sequencing Methods

11.2.1 Sequencing Platforms and Read Length

Massively parallel sequencers are often referred to as next generation platforms but already a further generation of single molecule sequencers that do not require a DNA amplification step, are becoming available. One of the most critical factors for virus detection is the available read length. Relatively short reads (50–100 bp) were obtained from first generation sequencers like Illumina's Genome Analyzer and similar read lengths were produced by Applied Biosystem's (Life Technology's) SOLiD. Shorter read length machines have particular value in other genomic applications like re-sequencing since, the proportion of reads that can be uniquely mapped to the human genome grows with the increase in read length but reaches a plateau after the first ~40 nucleotides (Whiteford et al. 2005).

However, the types of analysis discussed here require longer read length platforms and our discussion is limited to the use of these long-read machines. Roche's 454, and Life Technology's Ion Torrent offer 400 bp reads with new Roche chemistries extending this towards 800–1,000 bp. Of the single molecule sequencers, Pacific Bioscience's instrument offers ~1,000 bp read lengths on a circular template that is

re-sequenced multiple times to provide a consensus sequence of a single molecule. The studies reported below were conducted on Roche's 454 which is now being phased out. More recent platforms like Illumina's MiSeq offer appropriate read length with excellent depth of coverage and lower sequencing costs per base.

Three key steps are involved in obtaining the raw sequence on Roche's 454 sequencer family: generation of a double stranded DNA library by adapter ligation, emulsion based clonal amplification of the library and sequencing by synthesis. The last step utilises pyrophosphate release to drive the generation of a chemiluminescent signal, the record of a successful extension.

The DNA adaptors added during library preparation bind the library strands to complementary sequences on beads, with the aim of one strand per bead. The beads are then coated in an emulsion, containing reagents required for a PCR reaction, resulting in amplification of the original target on the bead by several million fold. After the emulsion is stripped from the bead and the beads are enriched for successful PCR reactions, they are deposited into individual picowells of the sequencing plate called a PicoTiterPlate or PTP. The wells are only 40 microns in diameter so only one bead is accommodated in each well. Further small beads are then added which contain the enzymes required for a pyrophosphate sequencing reaction. As one of the four dNTPs is added in a cycle, pyrophosphate is released into those wells incorporating the dNTP into the nascent complementary DNA strand. The released pyrophosphate activates luciferase resulting in a chemiluminescent signal which is detected in camera capable of resolving the signal from each well.

The Ion Torrent sequencer is also based on a high density array of microwells each of which incorporates a target sequence. As dNTPs are added, in a controlled cycle, their incorporation into the daughter strand of a particular target is detected through the associated release of hydrogen ions. Each well has an ion sensitive base layer overlying proprietary detectors, the strength of the signal detected being proportionate to the number of nucleotides incorporated in that cycle. Many of the features of the Ion Torrent, including the short preparation to sequence acquisition time, make this an attractive platform for viral sequence analysis. Because it relies on hydrogen ion release for detection rather than labelled dNTPs it can accommodate a wide range of library preparation methods.

Pacific Biosciences technology is a radically new system that enables single molecule real time (SMRT) sequencing by direct detection of nucleotide incorporation into the replicating nucleic acid strand. The key to the technology is the use of zero-mode waveguides (ZMWs). These are holes a few tens of microns in diameter in a fine metal film overlying a transparent silicon dioxide substrate. The target nucleic acid and DNA are immobilized in the bottom of the well. When a laser beam illuminates the wells through the transparent layer, the volume affected by the beam is limited to the bottom 20 zL of the well as, the ZMW is smaller than the cut-off wavelength of the laser light. In this miniscule volume, only the fluorophore labelled nucleotide being incorporated is activated by the laser beam. As the nucleotide is incorporated, the phosphate bonds linking the fluorophore are cleaved by the polymerase and a new cycle with a differently labelled nucleotide

can begin. The targets for sequencing are circularized DNA templates and the single circularized molecule is sequenced multiple times to provide a consensus sequence, reducing the overall error rate.

11.2.2 Errors in the Primary Data

Each of the systems has its own biases leading to sequencing errors. Both the SOLiD sequencer and Illumina's genome analyzers have been reported to under represent GC and AT rich regions with substitutions being the major error (Metzker 2010; Harismendy et al. 2009). For the 454 instruments the main problem has been insertions or deletions (indels) in homopolymer regions. In homopolymeric regions, multiple nucleotides are incorporated in each cycle; newer chemistry in which the signal is proportional for up to six nucleotides reduces the error rate and enables longer reads (Metzker 2010).

The vast majority of machine-specific sequence errors are removed during an important initial processing of the raw sequence data that accounts for these errors. The first step in this process is filtering to remove low quality reads revealed by base quality scores of the instrument. Other technical parameters like the light intensity of sequential pyrosequencing data can be used to asses quality, which often falls towards the end of the sequence.

Errors can be introduced into the library prior to the sequencing. Reverse transcription, and PCR have known error rates and selective PCR amplification could alter the frequency of variant sequences. These errors as well as the remaining known machine-specific error types can be detected after the reads are aligned with one another. For example, indels in homopolymeric regions seen in pyrosequencing, can be accommodated in the alignment of the reads by using reduced gap costs in those regions (Wang et al. 2007). A direct method of determining the errors due to reverse transcriptase and PCR steps can be achieved by tagging primers with a string of eight degenerate nucleotides that creates 65,536 distinct sequence combinations. Under conditions where each primer is used only once in reverse transcription, re-sequencing provides an indication of the error rate and the number of reads enables an accurate determination of variant frequency (Jabara et al. 2011).

11.2.3 Studying a Virus Stock by MPS

The "depth" or "coverage" of the sequencing run, which is the average number of times that a nucleotide is actually sequenced, varies based on the complexity of the source. Typically, a bacterial genome may be sequenced to a depth of 50 fold in a single run while for viral genomes the depth can be large, possibly 1,000–20,000 times, enabling the identification of variants or quasispecies within the population (Archer et al. 2012).

When investigating the genetic stability of virus seeds it is important to be able to define variant sequences and distinguish these from technical errors. In a series of 600 bp reads, with a 0.1 % error rate per nucleotide, 45 % of the sequences will have at least 1 error. Given the caveats described above, sequencing errors are largely randomly distributed and are assumed to be less common than valid single nucleotide polymorphisms. Clustering algorithms are used to define groups of common variant sequences or haplotypes within the population and the random errors removed (Zagordi et al. 2010; Quince et al. 2011); using a Bayesian approach, a posterior probability can be derived for each variant (Zagordi et al. 2010). Other methods of error correction involve multiple alignments (Salmela and Schroder 2011) which, while efficient, are computationally time consuming. All of these methods assume random distribution of errors but algorithms developed by Skums et al. (2012) allow for the non-random distribution of errors in homopolymeric regions as does the V-Phaser algorithm which distinguishes the co-variation between observed variants and process errors (Macalalad et al. 2012). After applying these error correcting procedures in control experiments, the limit of detection (LOD) of variants within the population is ~0.1–0.2 % and the limit at which variants can be quantitatively assessed is (LOQ) is typically at ~1 % (Tsibris et al. 2009; Maclalalad et al. 2012).

Once error corrected, each of the individual haplotypes can then be assembled into longer full length genome sequences. There may not be a unique solution to this global assembly if, there is less genetic diversity in the bridging regions linking variant sequences but algorithms have been developed that define the minimum number of global haplotypes that explain all the observed reads (Eriksson et al. 2008; Astrovskaya et al. 2011; Prosperi and Salemi 2012). For instance, if the sequence ABC has variants A' and C' it may not be certain in whether all 4 or, the minimum 2 haplotypes exist.

11.2.4 Two Ways to Use MPS for Detecting Adventitious Agents

Broadly, two types of MPS analysis are required in biosafety testing. The first is identifying replicating or, more challengingly, latent viruses within a cell substrate. In latent virus infections or, in infections where the viruses are defective and integrated, no virions are produced but latency associated transcripts and transforming genes, like polyomavirus T-antigen, may be expressed. In order to detect these sequences, the cellular transcriptome is sequenced and the viral sequences have to be detected amidst the far more numerous cellular sequences.

In a complicated source, like the entire transcriptome of a mammalian cell, one overnight MPS run will provide coverage of just over one. Nevertheless, housekeeping genes expressed at very low levels like GOLGA1 (<100 copies per cell) are detected and serve as internal controls of the sensitivity of the analysis. This "needle

in a haystack" problem is a complex bioinformatics challenge which requires the development of appropriate algorithms, as discussed below.

Moore and colleagues have identified several novel human tumor viruses using transcriptome analyses. They approximate that a single expressed transcript in a human cell occurs at a rate of about five per million, whereas, in their experience, novel viral transcripts are been seen at rate no lower than nine per million (Feng et al. 2008; Moore et al. 2011), thus setting a threshold for transcriptome biosafety testing.

The proper application of MPS must address sampling error and the statistical likelihood that a low incidence target, such as described above, will be seen by at least one read in a given sample size. The statistical argument is presented in Fig. 11.1. Here we assume that the typical mammalian cell contains 200,000 transcripts and are roughly the same size. If a million reads are collected and the human tumor virus occurs nine times out of a million, then the row labelled 1 in 200,000 best represents the statistics for detection of the rare event. Using one million reads, or a single plate on a 454 (PTP), has a confidence of 99.33 %; two PTPs has a confidence of $\sim$100 %.

The second type of analysis required in biosafety testing is detecting free virions in cell-free test articles. When evaluating raw materials like serum or screening the supernatant from a cell culture, the objective is to identify encapsidated viral sequences. The initial step is to reduce the complexity of the system by nuclease treatment and, after disrupting the capsids with chaotropic agents, the remaining DNA and RNA targets are amplified and sequenced. Low incidence sequences are easily seen in libraries built from low complexity sources, such as any cell-free material and fewer overall reads are required.

Virus seeds have low complexity but are sequence rich. Thus, adventitious agent testing of virus seeds resembles a transcriptome analyses rather than a cell-free analysis and sensitivity and read density are inversely related.

Unique mRNA to total mRNA ratio	Probability of obtaining at least one AA read (1 PTP)	Probability of obtaining at least one AA read (2 PTP)
1 in 1,000,000	63.21%	86.47%
1 in 800,000	71.35%	91.79%
1 in 600,000	81.11%	96.43%
1 in 500,000	86.47%	98.17%
1 in 400,000	91.79%	99.33%
1 in 300,000	96.43%	99.87%
1 in 200,000	99.33%	100.00%
1 in 100,000	100.00%	100.00%

Fig. 11.1 Confidence that adventitious agent (AA) low frequency events (*column 1*) would be detected using one (*column 2*) or two (*column 3*) plates (PTP) on the Roche 454 GS FLX

11.3 GMP Massively Parallel Sequencing

11.3.1 Overview

MPS is a complex procedure involving myriad devices and many steps starting with nucleic acid extraction from different matrices and ending with automated bioinformatic analysis. Implementation of MPS as a GMP process requires attention to the same requirements required of other analytical tests: accuracy, precision, (repeatability, intermediate precision), specificity, detection limit, quantitation limit, linearity and range, as well as robustness i.e. the response to deliberate small alterations in the test method. However, for most of MPS applications, robustness, specificity and detection limit are the most important attributes.

MPS assays extend well beyond the molecular biology bench top. As such, we defined MPS validation to be composed of three components: equipment including computer system validation (CSV) and 21CFR11 compliance (platform), laboratory processes (platform-specific 'modules') and data reduction (bioinformatics).

11.3.2 Validation of the MPS Platform

Our approach to validating an MPS platform included CSV for all PCs connected to data generating devices as well as establishment of a 21CFR Part 11 compliant environment for data security and data transfer integrity. The development and execution of the validation plan included user requirement specifications (URS), system risk assessments (SRA), installation qualifications (IQ), operational qualifications (OQ), and performance qualifications (PQ) for every piece of instrumentation that was deemed critical to the MPS workflow.

The data intensive nature of the MPS workflow necessitates viewing the process as a hybrid workflow/dataflow, and requires particular attention to 21CFR11 compliance and CSV requirements. 21CFR11 compliance gap analysis was performed on all systems, including vendor-supplied CSV packages, and documented procedural controls were implemented, as required, to maintain compliant data tracking (audit trails), archiving and retrieving. This comprehensive approach, including the validation of laboratory processes and data reduction discussed in detail below, ensured that the full MPS workflow, from instrument installation to final data output and analyses, met or exceeded cGMP requirements.

11.3.3 Validation of MPS Laboratory Processes

We designed the validation of the MPS laboratory process to reflect how MPS is conducted: in modules (Fig. 11.2). Since individual laboratory modules are selected

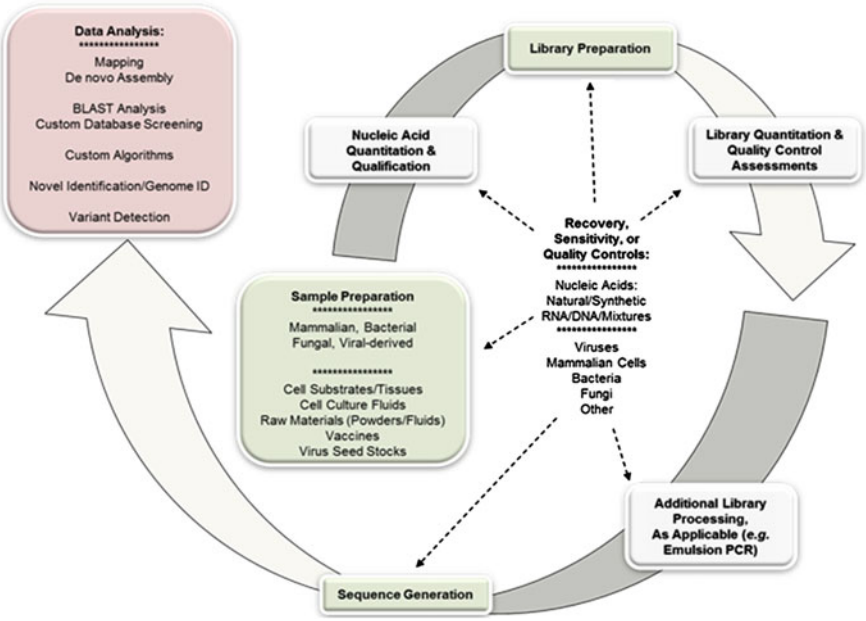


Fig. 11.2 Modular nature of MPS platforms. Core modules include sample preparation, nucleic acid quantitation and qualification, platform-specific library preparation and quality control assessments, additional library processing steps as applicable, sequence generation, and finally data analysis. Relevant process controls such as nucleic acid or viral 'spikes' may be introduced at any point (or module) of the process to assess critical performance metrics, an essential feature for validation of the system

and strung together based on matrix type, sample complexity and type of analysis, it was important that each module's validation be designed to stand alone. Furthermore, certain features of GMP validation, such as robustness, are not viable at the total system level and are best addressed as modules.

The laboratory core modules included sample preparation: nucleic acid isolation, library preparation, including platform-specific library processing steps, and sequence generation. Each module validation was designed to contain all of the relevant processes, quality control assessments and metrics (acceptance criteria) so that material of suitable yield and quality was generated. Though the modules were independently validated, each was broadly challenged to ensure that they could be integrated to accommodate diverse sample types and analyses.

Finally, we required that the validation plan bridge the modules by evaluating the complete assay for several key parameters. This was done first, by repeated sequencing of a reference *Escherichia coli* strain on different days with different operators and different reagent lots to provide data on accuracy, specificity and precision of the system. Second, we established the system sensitivity using a panel of controls spiked with known amounts of virus or viral sequences.

11.3.3.1 Selection of Control Materials

Samples used for biosafety testing range from cell substrates, culture fluids, bulk harvests, virus seed stocks, plasmid or vector preparations and raw materials. Although ideal, it is not feasible to validate every single material type. Therefore, a portfolio of relevant and well characterized materials were validated. Selection of 'real-world' control samples was based on a three key characteristics: (1) sample complexity—the total mass and sequence diversity of the nucleic acid strands in the sample, (2) sample volume—as a requirement for an adequate assessment, and (3) sample integrity.

In addition to these reference materials, a series of exogenous controls were spiked into one or more modules of the assay (Fig. 11.2) to track assay performance characteristics such as sensitivity and specificity. The intent was to provide the most comprehensive assessment and challenge to the system.

Spikes represented the range of expected biological attributes, for example, virion size, envelope type and genome type (RNA/DNA, single-stranded/double-stranded, small/large and circular/linear). Spikes consisted of defined synthetic or natural nucleic acid sequences such as purified viruses, characterized cell substrates (e.g. latently infected or chemically induced companion cell lines), or other biologicals.

11.3.3.2 System Contaminants

Understanding the intrinsic (contaminating) nucleic acid content of the system is crucial. For example, reagents, particularly enzymes used for sample processing and library preparation are often contaminated with nucleic acid fragments as by products of production. Even certified reference materials from reputable suppliers have been discovered to be misidentified after MPS characterization (Côte and Kolman, unpublished observation).

Animal derived materials, whether test articles or components of the assay matrix, are carriers of unanticipated and variable nucleic acid contaminants. It is vital to identify sequences that are intrinsic to control material or to the system itself in order to avoid misidentification in test articles.

11.3.3.3 Acceptance Criteria and Executing Module Validation

Acceptance criteria were defined to act as clear gateways of adequate and sufficient sample processing. By necessity, we used a range of acceptance criteria to accommodate the myriad sample types supported by a module. We also found it important to include tests to differentiate between system biases (e.g. system errors due to the nature of the sample processing or sequencing chemistry, or platform-specific methodologies) from true test failures.

11.3.3.4 The Assay Modules

Items validated in each module are outlined in Fig. 11.3. In general, the early modules encompass multiple optional processes which gradually coalesce into three identical procedures in the later modules that differ only by reagent volume.

The first module included multiple, parallel sample preparation methods for assay-specific nucleic acid isolation using customized or commercially available (kit-based) methods (e.g. viral nucleic acid-specific methodologies or total DNA- or RNA-specific kits). The isolation of RNA from cell substrates for transcriptome analyses is a relatively straightforward process whereas, isolation of encapsidated viral sequences from cell-free substrates, including raw reagents, poses unique challenges due to sample volume variability and potential biases in the efficiency of virus particle recovery.

The second module included quantitation and quality assessments of the highly selective nucleic acid populations isolated by module one. This included absorbance and fluorescence-based RNA and DNA-specific assays for concentration, purity and integrity determinations. This was followed by validation of multiple library preparation modules used for the conversion of the material into a sequencing-compatible double-stranded DNA library (e.g. standard 'shotgun' or RNA-Seq libraries, and customized amplicon- or other targeted-based methodologies).

Validation of the fourth, fifth and sixth modules was simpler as these were based on established methods using mostly commercially available kits and established processes. This included library quantitation using a robust and highly sensitive quantitative PCR assay, library amplification by an emulsion-based PCR, and sequence generation (the latter two validated using all available formats from Roche). The end result of this extensive process is absolute confidence in the quality and integrity of the raw sequence data for analysis.

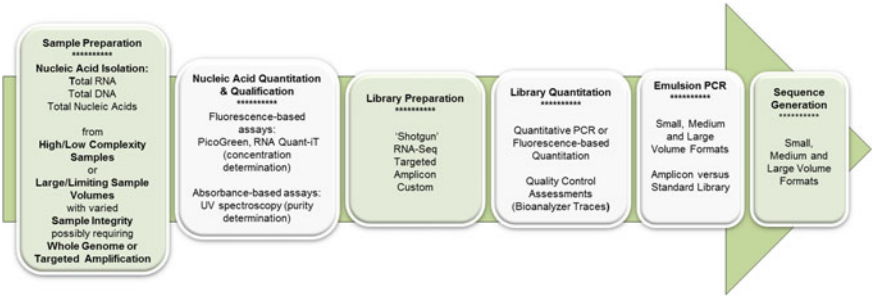


Fig. 11.3 Validation of custom and core modules on the Roche GS FLX/FLX+ Sequencer system. Each block represents a distinct module of the process and is illustrated in the order required to complete a sequencing run on the Roche system. All modules may be further divided into sub-modules for flexibility of the system and to address the varied nature of starting materials and desired level of sequence data generation. Any material generated from a module that meets all acceptance criteria is then used for subsequent module validation

As advancements in technologies and chemistries are made or, as improvements and new modules are identified, this validation approach will continue to expand. Continued validation of methods tailored to specific sample types is required to ensure optimal recovery of the sequences of interest. Furthermore, use of an appropriate set of controls is absolutely essential for a robust validation that can be applied to the broadest range of input materials.

11.4 Algorithmic Methods to Identify Viruses

As mentioned previously, MPS methods shift the burden of identification from the design stage of the assay to the data reduction stage. As a result, a whole new aspect of performance control and validation are needed. The process of MPS data reduction is described below. The control structure and validation program is then discussed in context.

11.4.1 The Basis of Read Identification

The objective of data reduction is rapid and accurate sequence identification with minimal downstream data manipulation. This precludes the use of short-read platforms which require de novo read self assembly, prior to comparison with a database.

One-step read identifications are made using a public, version controlled, software tool maintained and provided by the National Center for Biotechnology Information (NCBI) called the Basic Local Alignment Search Tool or BLAST. The tool can used remotely, at the NCBI website, or can be downloaded and run on a local host (Altschul et al. 1990).

BLAST accepts input sequences or "queries" and compares them to a specified NCBI or local database of "known" sequences. There are several different kinds of BLAST algorithms that have different purposes and different capabilities. They are named as variants of the term BLAST and are listed in Table 11.1. For example, if one is comparing a newly sequenced human genome with the NCBI human reference, the appropriate algorithm is megablast since the two will be practically identical.

BLASTN is the best choice for virus detection since it is designed to identify similar sequences rather than nearly identical sequences. BLASTN offers another accommodation for virus hunting—a small Word Size trigger. All BLAST identifications start with an initial scan that looks for a short, perfect match between contiguous bases in the query and an element in the database. One can think of it as the nucleation site for the sequence identification. The required length of this initial match is the Word Size. The Word Size match becomes the site from which a broader, possibly much weaker, sequence homology is revealed. Conversely, no

Table 11.1 NCBI BLAST algorithm and a few key parameters

Program	Purpose	Word size
Megablast	Identify the query sequence	16, 20, 24, 28, 32, 48, 64, 128, 256
Discontiguous megablast	Find sequences similar to query sequence	11, 12
BLASTN	Find sequences similar to query sequence	7, 11, 15
Translated BLAST (tblastx)	Find similar proteins to translated query in a translated database	

Taken from NCBI help

BLAST identification is made if there is no sequence identity of length specified by the Word Size. Finally, the longer the Word Size, the greater the expectation of identity between query and database; short Word Size allows for greater sequence divergence between query and database.

All BLAST analyses produce several measures of the quality of a sequence match or "hit". The most common of these is the e-value. The e-value is a measure of the chance that the sequence hit in the database could have been generated at random in a database of the given size. Though we limit our analyses to hits that have e-values of 10^{-3} or less, the analysis can be done with reduced stringency. Since FLX/FLX+ reads are so long, this has no impact on the identifications made.

The other common measure of the quality of a hit is the score. The score is a measure of the quality of the match and is influenced by mis-matches, insertions and deletions (indels) and, importantly, by the length of the match. Scores of short reads deteriorate quickly as mis-matches and indels collect, to the point that they are difficult to discern from non-matches.

Long reads counter-balance mis-matches and indels, such that scores remain high and identifications can be made above background. One-hundred base reads can suffer a base change approximately once every five bases and still make an identification; for 300 base reads, the rate is a base change every three bases. Eight hundred base reads will extend accuracy of detection even further.

The concept is illustrated in Fig. 11.4 using two parallel BLAST analyses. An artificial parental sequence and a series of progressively more degenerate relatives were generated to provide a continuous series from 99.20 to 55.90 % identity. The percent match is listed as the first four digits in column two, "Description". In panel A, 300 base reads are BLASTed and clear matches to the parental sequence are seen down to the strand with 66.60 % homology. In this case, the score of 156, column 3 "Max score", is easily distinguished from low level sporadic matches in the strands with less homology. In panel B, the same test is done using 100 base reads of the same starting sequences and the last clear identification is made with a score of 145, corresponding to only 84.6 % identity.

(a)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
22182	99.20 1000	450	450	100%	8e-131	93%
22183	84.70 1000	354	354	100%	5e-102	86%
22184	73.90 1000	264	264	100%	6e-75	80%
22185	70.10 1000	284	284	99%	7e-81	81%
22186	68.20 1000	197	197	99%	8e-55	75%
22187	66.60 1000	156	156	93%	2e-42	73%
22188	65.30 1000	98.7	98.7	93%	6e-25	68%
22189	64.00 1000	96.9	96.9	94%	2e-24	67%
22190	63.50 1000	107	107	95%	1e-27	68%
22191	61.80 1000	82.4	82.4	75%	4e-20	68%
22192	60.00 1000	73.4	73.4	75%	2e-17	67%
22193	58.20 1000	50.0	50.0	30%	3e-10	72%
22194	55.90 1000	42.8	42.8	10%	4e-08	90%

(b)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
62416	99.20 1000	187	187	100%	5e-52	94%
62417	84.70 1000	145	145	99%	2e-39	87%
62418	73.90 1000	113	113	99%	1e-29	81%
62419	70.10 1000	129	129	95%	1e-34	84%
62420	68.20 1000	105	105	85%	1e-27	83%
62421	66.60 1000	84.2	84.2	58%	5e-21	86%
62422	65.30 1000	59.0	59.0	53%	2e-13	80%
62423	64.00 1000	60.8	60.8	66%	5e-14	77%
62424	63.50 1000	69.8	69.8	72%	1e-16	77%
62425	61.80 1000	53.6	53.6	90%	8e-12	71%
62426	60.00 1000	53.6	53.6	90%	8e-12	71%
62427	58.20 1000	44.6	44.6	57%	4e-09	74%
62428	55.90 1000	42.8	42.8	25%	1e-08	90%

Fig. 11.4 A study of the ability of BLAST to identify unknown (degenerate) sequences. *Panel A* is a re-construction using 300 base reads; *Panel B* uses 100 base reads

11.4.2 Databases

Database resources are critical for the successful identification of unknowns. Public databases are comprehensive collections of sequences and are easy to access, but there are hidden pitfalls. Some are listed in Table 11.2.

None of the issues listed in Table 11.2 prohibit the identification of true viral signatures. However, they can delay and add unnecessary confusion to automated systems and regulated operations. For these reasons, databases become curated or modified. In our case, none of the confounding sequences are removed from our curated viral database, but we tag them with a note that describes the issue so that mis-identifications are avoided.

Table 11.2 Examples of confounding annotations in viral sequences

	Detection	Actual
Not uniformly annotated	"Parvovirus" versus "parvo virus"	
Under-annotated	BLAST for "parvo"	Hit: "Ureaplasma urealyticum strain 67 MB multiple banded antigen"—a bacterium
Correctly identified but non-viral	AF104019 Bovine viral diarrhea virus-2 subgenome A polyprotein mRNA, partial cds	Includes host ubiquitin gene
Incorrectly identified	Stealth virus	Typically bacterial, no viral matches

11.4.3 The Algorithm

Biosafety testing and adventitious agent detection can suffer neither false positives nor false negatives. At the same time, multi-day computational analyses must be performed efficiently, so that problems can be identified and remediation begun as soon as possible. For this reason, the BioReliance Reliant Algorithm™ for adventitious agent detection uses multiple, sequential redundant BLAST analyses. The process is illustrated in Fig. 11.5 with the bounded area in red being subject to

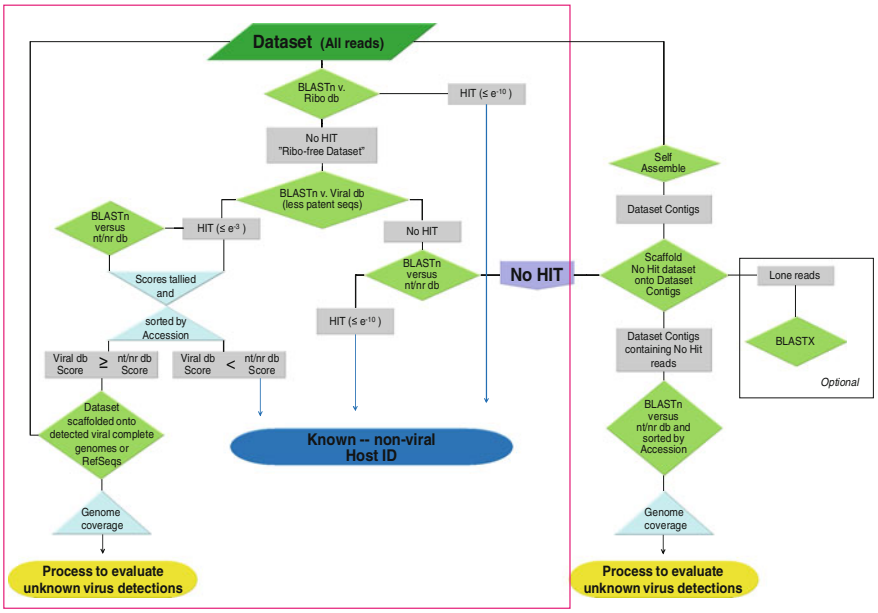


Fig. 11.5 Outline of the Reliant Algorithm™. The area bounded by the *red box* is subject to validation whereas processes outside the *box* are investigational

validation with the areas outside forming investigational procedures. The key advantage of long reads is that assemblers are not necessary to obtain an identification avoiding the complexity of validating their use.

The first analysis is versus a ribosomal sequence database. The intent is to remove ribosomal sequences from the MPS dataset so that it can be analyzed more quickly. Ribosomal sequences are well conserved as a whole, and bear no resemblance to viral signatures.

In the second analysis, the ribosomal-free sequences are BLASTed versus the curated Viral database. Sequences that "hit" are considered putative viral and further challenged to ensure they are not false-positives. The challenge is to BLAST them versus GenBank nr/nt, the master repository of all nucleotide sequences. At a minimum, the queries that hit the Viral database will hit the same sequence in GenBank nr/nt with the same score. In this case, the identification as a viral signature holds. The other possibility is that the query hits a non-viral sequence in GenBank nr/nt with a higher score that it hit the Viral database. In this case, the identification fails—it is a false positive.

In the second analysis, ribosomal-free sequences that do not hit the Viral database are challenged to ensure they are not false-negatives. The challenge is also versus GenBank nr/nt, but here, a match represents confirmation that the query is either a host sequence or perhaps, a trace contaminant. If no match is made to GenBank nr/nt, we have a putative false-negative.

Apparent false negatives are of two kinds—short and long. As discussed, short reads are difficult to identify because of their length and may be artifacts. Long reads are true false-negatives and represent genomes that may be less than 66 % homologous to a known viral genome.

In order to confirm the false-negative as viral sequences, all reads in this dataset are subjected to self assembly. Assembly is an important tool in unknown identification because it permits extremely long artificial sequences—contigs—to be built from raw data. As the contigs get longer, the more they can diverge from a known sequence and still support a statistically significant identification. This is exactly the same principle outlined above. The problem is that assemblers are idiosyncratic; two assemblers can give dramatically different results starting from the same dataset. It is for this reason that we do not assemble raw data until it is absolutely necessary.

The expectation is that contigs will permit an identification of most unknowns, even confirmation that a false negative is a new virus. But there will always be unidentified reads. Many will be short—and some will be sequencing artifacts that are unidentifiable.

The last chance for identification of reads such as these is tblastx, in which nucleotide queries are translated in all six frames into the amino acid equivalent and then BLASTed versus a protein database. The intention is that converting the simple nucleotide code into a high-complexity code, with internal redundancy, can result in a definitive call. Unfortunately tblastx analyses are extremely processor intensive and, typically, the results are difficult to interpret.

The output of the Reliant algorithm is a list of queries that are virus signatures. It is left to expert virologists to conclude whether the signatures are sufficient to warrant an orthogonal analysis in search of an active infection.

11.4.4 The Algorithm, Automation, Performance Control and Two Levels of Validation

While the logic of the Reliant Algorithm™ can be listed as a step by step procedure, the requirement is that this process, like all of the laboratory processes, must be performance controlled. Since the algorithm is executed entirely through software, its operational execution requires CSV and a 21CFR Part 11 compliant environment. Moreover, as the software is entirely custom and internally authored, it must be validated and version controlled. This requires compliance with and validation to standards established in Software Development Life Cycle (SDLC) control SOPs.

Validation of the Reliant Algorithm™ was performed according to SDLC SOPs. The result provides confidence that the operation can be performed reproducibly and will generate the same result within specific tolerances.

What is not tested in this process is the ability of the algorithm to identify unknowns of specific levels of sequence divergence. This was validated as a robustness test by using the algorithm to identify a modified, known virus. This is illustrated in Fig. 11.6 for poliovirus. The poliovirus genome was randomly mutated several thousand times and then randomly sheared to defined lengths several thousand times. Each of these mutated, sheared genomes was run through the Reliant Algorithm™. The data reinforced the importance of read length in obtaining an identification and allowed us to establish confidence limits on the ability of the algorithm to detect diverged genomes at a defined percent identity and genome size. For a given mutation rate base substitutions and indels were incorporated at a ration of 4 to 1, in line with published data for viral variants (Sanjuán et al. 2010). However, an overall mutation rate of say 30 % is a much stricter test of detection than the real situation as, variant viruses usually display areas of conservation in some non-structural genes with hypervariation in capsid or envelope protein genes.

11.4.5 Comparability, Community, and Proficiency Testing

The safety of vaccines and biologics is preeminent. Performance of biosafety assays in different locations must be controlled and the results across sites must be equivalent. But, as with diagnostic tests for clinical use, different labs will implement assays in different ways. Complex assays such as MPS and associated data reduction are clearly more difficult to standardize than an off-the-shelf QPCR assay. Furthermore, MPS datasets are not terminal. They are neither destroyed upon

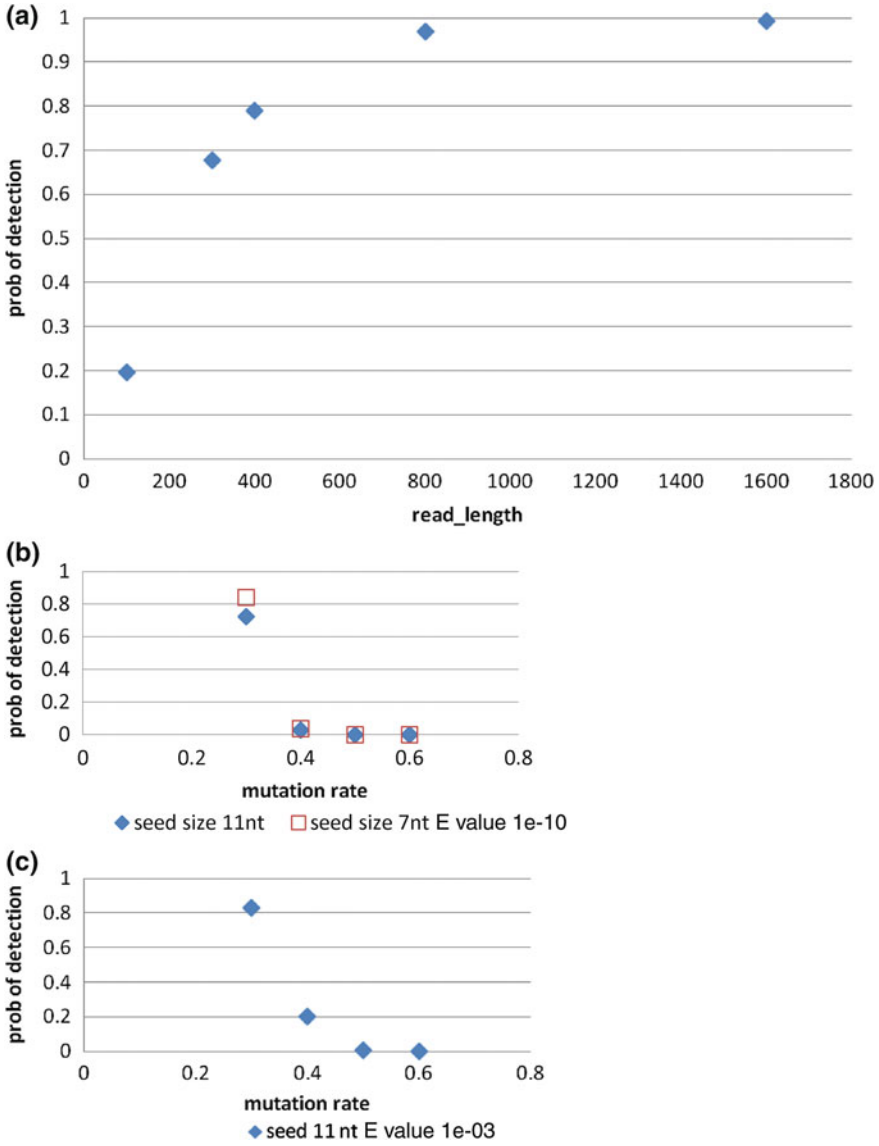


Fig. 11.6 **a** Probability of detection of a variant poliovirus with an overall mutation rate of 30 % (24 % base substitutions 6 % indels). *BLAST parameters* seed size 11nt, and E-value 1e-10. **b**, **c** Illustrates the effect of word size and E-value on detection for a randomly sheared 300 bp sequence of a mutated poliovirus genome with 4 to 1 ratio of base substitutions to indels

analysis nor modified while detecting limited targets, for example, like QPCR and infectivity assays. MPS datasets can be revisited when new viruses enter public databases.

A good solution is to require periodic proficiency testing of providers of MPS assays for adventitious agent detection using standardized controls. Data should be submitted to regulators in a format that permits analysis by publically available algorithms to ensure uniformity in analysis. Admittedly, solutions such as these require considerable discussion and the support of the commercial and regulatory communities.

11.5 Application of Sequencing in Virus Discovery and Vaccine Development

11.5.1 Virus Discovery

The advent of massively paralleled sequencing technology has initiated a renaissance in the discovery of micro-organisms resulting in the identification of new pathogens and new genera of viruses (Li et al. 2010, 2011a, b; Phan et al. 2012). The power of MPS in identifying etiological agents was illustrated by the identification of a new arenavirus in a transplant recipient, through unbiased high-throughput sequencing, after it had eluded detection by PCR and microarray strategies by Palacios et al. (2008). The rate of development is exemplified by the discovery of new human polyomaviruses. Just over a decade ago only BK and JC viruses were recognised as human polyomaviruses. With the discovery of MW polyomavirus (Siebrasse et al. 2012), the number stands at 10 with the Merkel cell polyomavirus linked to the carcinoma of the same name (Feng et al. 2008).

In some cases, single sequencing runs can identify multiple new viruses. In fecal samples the interpretation of the data can be challenging because, in addition to bacteriophages, viral sequences from animal or plant food may be present (Li et al. 2010, 2011a, b; Kapoor et al. 2011). This problem is illustrated by the recent finding of a new parvovirus genus, in 4 % of rotavirus negative diarrhoea cases in African children (Phan et al. 2012). This new virus is likely to be the aetiological agent of the diarrhoea but additional prevalence data and serological data will be needed to confirm that.

MPS is being used in diagnostic settings where a significant proportion of cases do not have a known etiological agent, like childhood respiratory disease. One of the problems of MPS analysis from nasopharyngeal swab samples and tissues is the high content of ribosomal RNA which can reduce the sensitivity of detection of RNA viruses. One solution is to reduce the background by priming with hexamers that do not anneal to rRNA sequences (de Vries et al. 2011). Other clinically challenging areas that are being subjected to MPS approaches include hepatitis cases with no known viral etiology and unexplained Dengue-like febrile illness where pathogens remain unidentified in 40 % of cases (Yozwiak et al. 2012). The wave of discoveries arising from MPS will inform diagnostic and clinical approaches to disease and fuel the pipeline of vaccine candidates.

11.5.2 MPS in Vaccine Research and Development

Vaccines against monotypic viruses like measles virus have been amongst the most successful but there are continuing challenges in developing vaccines against viruses that display frequent variation like influenza virus, which undergoes significant antigenic shift through genetic reassortment and antigenic drift through mutation. Manfred Eigen and colleagues first formulated the concept that variation in a population of RNA viruses could be conceived as a cloud of variants or, quasispecies, and was inherent due to the absence of the proof reading capacity of RNA polymerases (Eigen and Schuster 1977; Biebricher and Eigen 2006). This concept was supported by pioneering analysis of variation in replication of RNA molecules (Domingo et al. 1978). Mutation rates of RNA polymerases have been estimated at 10^{-3} and 10^{-5} mutations, per nucleotide, per replication cycle. At slightly higher levels of mutation all information content would be lost so these viruses can be viewed as evolving at the edge of chaos (Domingo et al. 2006). Inherent in the initial concept of a quasispecies was that the ensemble of quasispecies, rather than individual viruses, formed the replicating unit; an hypothesis that leads to the prediction that populations with a restricted fitness can outcompete those with a wider range of fitness values. The evidence for this formal definition of a quasispecies has come under criticism (Holmes 2010) and it is notable that the term is often more loosely used simply to refer to intra-population variation.

Antigenic change has proved to be a particular challenge in the development of certain vaccines. Escape mutants to both neutralising antibody and CD8+ T cell responses have been well documented in HIV infections (Borrow et al. 1997; Price et al. 1997; Richman et al. 2003; Allen et al. 2005; Fischer et al. 2010; Haynes et al. 2012). The introduction of MPS has enabled the evolution of immune escape variants to be monitored during the course of an infection. Henn et al. (2012) showed that escape from the immunodominant Vif B38-WI9 and Nef A24-RW8 epitopes of HIV, occurs at rates of just under 0.1 day^{-1} and by day 59, 56.6 % of the viral population expressed one of four intra-epitope variants of B38-WI9. Similar analyses of neutralizing antibody epitopes reveal that low level neutralizing antibody develops as early as 2 weeks and selects for escape variants (Bar et al. 2012). MPS enabled identification of the full sequence of transmitted-founder viruses and their trajectory of escape from neutralising antibody. Both the initial monotypic neutralizing response and the pattern of escape varied between individuals. In one subject the initial antibody response was to V1 region of the envelope glycoprotein but, by day 16 post sero-conversion, V1 variants were detectable in the virus population. These may have been driven by APOBEC as the neutralizing antibody region was enriched for mutations at APOBEC motifs (Bar et al. 2012). Effective immunisation strategies for HIV are going to be dependent on an understanding of the pattern of immune escape that MPS is revealing.

Similar MPS data for other viruses is providing important information about the evolution of virus variants during infection (Cordey et al. 2010; Parameswaran et al. 2012; Tapparel et al. 2011), the development of drug resistance (Verbinnen et al. 2010;

Ghedin et al. 2011; Fonseca-Coronado et al. 2012; Jabara et al. 2011; Svarovskaia et al. 2012), the diversity of antigenic variants (Bull et al. 2011; Höper et al. 2012) and the risk of generating new pandemic viruses (Russell et al. 2012). In the latter case, recent work on avian A/H5N1 influenza viruses has shown that as few as five amino acid substitutions, four with reassortment, might result in mammal-to-mammal transmission. MPS of avian influenza virus populations indicated that two of these substitutions are common in avian A/H5N1 viruses (Russell et al. 2012).

11.6 Application of MPS in Quality by Design Strategies for Raw Materials

The majority of contamination problems in the biotechnology and vaccine industry have a root cause associated with adventitious agents in raw materials. In animal origin free systems plant derived materials, like peptones, need to be considered as possible sources of contamination by a range of agents including, spiroplasmas and animal viruses derived from fertilizers.

As the biotechnology industry matures, there is increasing emphasis on QbD principles as formulated in ICH guidance document Q8 (R2) Pharmaceutical Development (2009). Encompassed within QbD is a control strategy designed to ensure that a product of required quality will be produced consistently. Elements of the control strategy focus on input materials and the "design space" that affects control of those materials. The difference between the traditional approach and a QbD approach to raw materials is worth examining; fetal bovine serum (FBS) is used in the example below but the principles apply to all raw materials.

An inherent part of traditional testing strategies was the belief that it was possible, with a high degree of certainty, to select sera free of adventitious agents and that if a material passed a Code of Federal Regulations (9 CFR Section 113.53) or, CPMP test, it was safe to use. A typical batch of FBS can consist of one to two thousand, individual, 1 L samples. Consequently, certain viruses like Bovine Viral Diarrhoea virus, which occurs as a persistent infection in 1 in 100–500 fetuses, is present in the majority of batches. In contrast, other virus infections, particularly those transmitted by arthropods, are only sporadic. This is well illustrated by *Cache Valley virus* contamination of US origin FBS. Four major episodes of fermenter contamination by this virus have been recorded (Onions 2004; Nims et al. 2008). While contaminations by this virus are uncommon, they are very serious as *Cache Valley virus* (CVV) is a zoonotic virus associated with fatal encephalitis. Contamination by CVV reveals the limitations of standard serum testing. Detailed analysis of one episode indicated only 10–100 virions entered the fermenter in 20 L of serum indicating that it was unlikely to be detected by testing a standard 50–100 mL of the main pool (Onions 2004).

Many of the assumptions about the frequency of particular viruses in serum have had to be radically revised following the introduction of MPS. Allander et al. (2001)

first applied this approach to bovine serum resulting in the surprising discovery of two new bovine parvoviruses BPV-2 and BPV-3. More recent studies using MP-Seq™ confirmed these findings and resulted in the finding of a new parvovirus BAAV-2, a member of the dependovirus genus (Onions and Kolman 2010). As discussed below, these are very frequent contaminants of serum and parvoviruses are amongst the most resistant viruses known, posing a challenge for inactivating procedures. Little is known of the tropism of BPV-2 and 3, even within their host species, but this family of viruses have shown major changes in host range. The onset of the *Canine parvovirus* pandemic around 1979 is believed to have followed cross transmission of a feline virus following three mutations in the capsid gene. In contrast, BAAV-2 and possibly the other bovine dependovirus BAAV-1, has a wide host range with BAAV-2 able to infect human cells.

New parvoviruses were not the only surprising discoveries. In a survey of four different FBS serum lots from major manufacturers, 2 out of 4 batches had complete sequences of bovine noroviruses and 2 also had sequences of kobuviruses (Onions 2011). In both cases it was possible to reconstruct the complete genomes of these viruses and, as the samples had been nuclease treated, these genomes were contained within capsids and therefore potentially infectious (Fig. 11.7).

The greater understanding of viruses present in serum that has come from new technologies like MP-Seq, emphasises the need for a quantitative risk based approach.

A new approach to raw material quality control involves three or four steps:

1. Understanding the universe of potential contaminants in the raw material.
2. Developing specific, quantitative, assays for those viruses, taking account of the statistical limitations of sampling from the raw material pool.
3. Relating the potential viral load in a given batch of raw materials to inactivating procedures like gamma-irradiation or, high temperature short time (HTST).
4. Where no inactivating steps are in place for the raw material, adding monitoring assays later in the process to ensure the viruses are eliminated.

Understanding the range of contaminants that may be present is best determined through the use of new technology like MP-Seq™ that makes no assumptions about the nature of the virus (or other biological contaminant) or, its ability to replicate in a set of pre-determined indicator cells. MP-Seq™ is not likely to become a routine batch by batch quality control tool until sequencing costs fall further. However, several manufactures are now embracing the concept of reviewing the data from MP-Seq™ on several batches of raw materials from a given supplier. This approach should be linked to agreements that tightly specify the geographical source of the materials so that the MP-Seq™ data are reflective of the universe of contaminants from that supply source.

As discussed above this technology provided indications that new viruses like BPV-2, BPV-3, BAAV-2, *Bovine norovirus* and *Bovine kobuvirus* were frequent and often high level, contaminants of serum. The next stage is to develop specific assays for these viruses. In the case of BPV-2 and 3 permissive cell systems have not been identified and therefore specific PCR assays have been used to determine

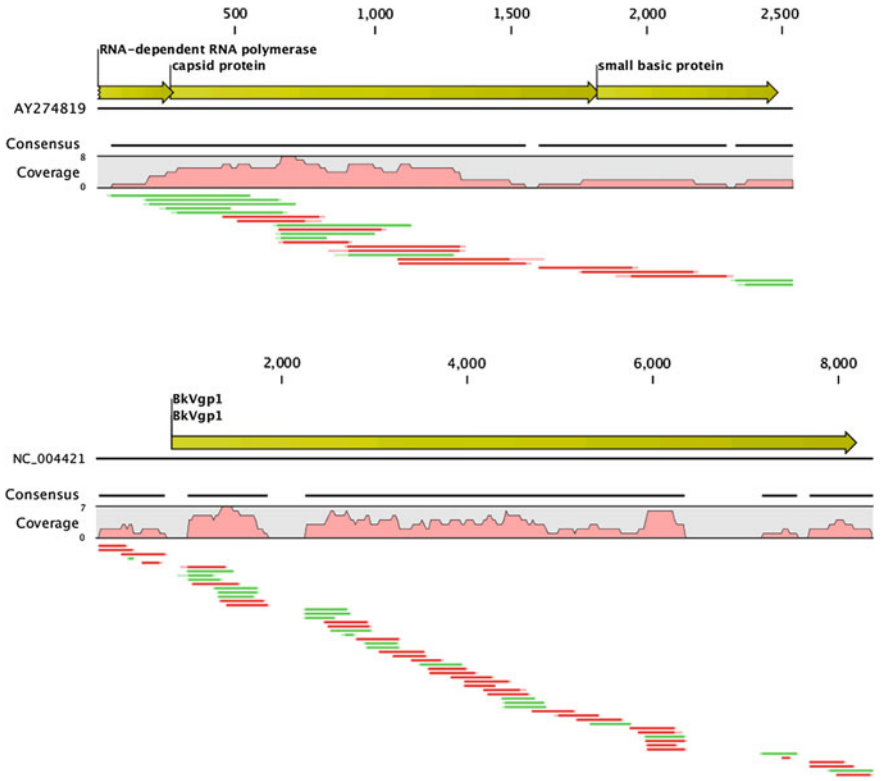


Fig. 11.7 Detection of bovine norovirus (*top*) and bovine kobuvirus (*bottom*) in bovine serum pool using by MP-Seq. Individual sequences forward in *green*, reverse in *red*, are shown scaffolded against a reference sequence

the frequency and level of viral genomes in serum. Quantitative PCR analysis has indicated that the encapsidated genome count is variable but can be very high, up to 10^4 ge/ml for BPV-3.

Finally, the viral load should be linked to inactivating procedures. In Europe it is now a requirement to use gamma-irradiated serum in, the manufacture of veterinary medicinal products but in a QbD approach it is important to understand the limitations of inactivation by irradiation. Standard irradiation involves treatment with 30 kGy, but where batch irradiation is used, outer parts of the batch may receive higher doses impairing the quality of the serum. The kinetic inactivation curves for gamma irradiation are essentially first order. The dose required to produce a 1 log10 inactivation of the virus, or D value, varies between viruses but lies in the range of 3.9–5.3 kGy for several major groups (Sullivan et al. 1971). Protection in a serum environment is likely to increase protection for viruses and, as Plavsic and Bolin (2001) demonstrated, ssDNA viruses like circoviruses and parvoviruses are remarkably resistant to irradiation. This has important consequences for analysing

FBS which may contain BPV-2 and BPV-3 genomes at levels above the capacity of irradiation to inactivate. An appropriate approach is to screen batches by quantitative PCR using only those batches with a low level of genomes. For instance, the control might specify an inactivation capacity 3 log10 greater than the virus load.

Where no serum inactivating steps are in place then, as part of the QbD approach, appropriate in process tests should be conducted. An evaluation of the capacity of a downstream purification process to inactivate or remove the contaminants identified in serum should also be undertaken. Implementation of this approach would have avoided the catastrophic contamination of rotavirus vaccines by porcine circoviruses introduced in contaminated trypsin (Victoria et al. 2010).

11.7 Characterizing Cell Substrates by MP-Seq

11.7.1 Cell Identity and Cell Productivity

Characterising cell lines involves two key elements, verifying the identity of the cells and ensuring their freedom from adventitious agents. The former requirement comes from the known history of mis-identified cells, particularly HeLa being deposited in repositories. However, the problem still exists (Nardone 2008) and newer methods, like analysis of microsatellite short tandem repeats (STR) markers with high heterozygosity values, are being used to identify human and simian cells in place of older methods like isoenzyme profiling (Almeida et al. 2011; Eltonsy et al. 2012). Sequence analysis of STR markers or, mitochondrial D-loop sequences, can also provide data on the genetic stability of the cell line on passage.

Additional data from MPS based transcriptome analysis can supplement data on identity and can be used to provide information on important alleles like the PRNP gene in human cell lines. Sequencing the PRNP genes or, their transcripts, can be used to exclude mutations associated with familial forms of transmissible spongiform encephalopathy or polymorphisms like $129^{met/met}$ associated with a higher risk of vCJD/BSE transmission (Lloyd et al. 2011).

One of the most exciting applications of MPS is in identifying gene expression or miRNAs signatures associated with highly productive cells. These data can be used to select clones or, to engineer cells using zinc finger technology that permits precise, high efficiency, knock out or insertion of genes (Klug 2010). Several classes of interrelated targets may enhance performance:

- Genes involved in the antiviral response particularly interferon and interferon stimulated genes (ISGs).
- Genes regulating apoptosis.
- Genes affecting viral entry or replication.

In Vero cells, which display defective interferon induction (Mosca and Pitha. 1986) other stress response genes including heat shock proteins and genes associated

with the oxidative stress response are upregulated and these may be amenable to engineering (Vester et al. 2010). MDCK cells do produce class I interferons on infection by influenza virus but the main antiviral proteins that affect influenza replication in other cells, Mx1 and Mx2, do not appear to very effective in canine cells (Frensing et al. 2011) although, a transfected canine Mx2 gene but not Mx1, has activity against VSV in murine cells (Nakamura et al. 2005). Overall the interferon response in MDCK cells has not been shown to limit influenza virus titre Seitz et al. 2012).

Apoptosis of virus infected cells is a potential defence mechanism reducing virus yield from infected cells and in response some viruses have evolved or, captured cellular, anti-apoptotic genes. The position can be complex with apoptosis late in viral replication being a method to enhance virus release. Similarly other mechanisms leading to cell death like autophagy can be an important part of the replication strategy of certain viruses (Meng et al. 2012). In addition, other targets in the apoptotic pathway may be applicable for certain virus vaccines. For instance, the CCCTC-binding factor (CTCF) and the EGFR-coamplified and overexpressed protein genes (ECOP) are down regulated by a micro-RNA in infected cells. Transfection of these genes into cells can enhance the production of West Nile Fever virus (Smith et al. 2012).

11.7.2 MP-Seq™ of Cell Substrates to Demonstrate Their Freedom from Adventitious Agents

11.7.2.1 Exogenous Viruses

The ability to detect latent viruses and defective transforming viruses, as well as replicating viruses, is the key attribute of MP-Seq™ analysis. With the exception of dependoviruses, a latency associated transcript or, transforming gene, like the T-antigens of polyomaviruses, are expressed in infected cells. A transcriptome analysis is conducted in parallel with an analysis of the supernatant media which confirms the presence of replicating viruses and excludes viruses that are present in the media but have not infected the cell line.

Another key attribute of MP-Seq™ analysis is the ability to detect viruses in cell lines where there is little or no genomic sequence data. This is exemplified by the analysis of insect cell lines. For instance, MPS methods have been used to detect a novel rhabdovirus in the widely used Sf9 cell line (Ma et al. 2014). Similarly, in our validation of MP-Seq™ we analysed a *Trichoplusi ni* cell line (BTI-TN-5B1-4 "High Five™"; Invitrogen), known to contain a sub-clonal infection with an alphanodavirus (Onions et al. 2011). A total of 468,579 reads were recorded in untreated High Five™ cells and 365,299 in heat shocked cells which, after removal of ribosomal RNA sequences, fell to 207,419 and 131,051 respectively. Of these, 470 reads from the untreated cells and 326 from the heat shocked cells were

recorded as unique hits against our virus sequence database. As for mammalian viruses, an algorithmic approach was required to filter out false hits. For instance, multiple hits against baculovirus genomes were recorded, but these were to a transposable element, *piggyBac*, found in baculoviruses and expressed in *T.ni* cells.

These hits enabled complete reconstruction of the total bipartite genome of the alphanodavirus. The complete sequence of this virus was already known so to test the capacity of the method to detect unknown viruses, this sequence was removed from the curated database. Scaffolding the sequences against other nodaviruses and assembly of contiguous sequences enabled the complete genome of the virus to be reconstructed with ease. For RNA2, which encodes the capsid, the intact genomic sequence appeared to be a minor population with the dominant species containing deletions. This partial defective genome may account for the very low frequency of intact virions observed in this cell line. However, insect cell lines have another mechanism, RNA interference (RNAi), that ensures a high frequency of silent infections. Dicer acts as the sensor recognizing and cleaving dsRNA into 22 bp length siRNA fragments, these are then used by the effector Argonaute protein to silence the target viral RNA (Wang et al. 2006). Consequently, for insect cell lines it may be useful to supplement standard transcriptome analysis with specific sequencing of siRNAs, an approach that led to the finding of four new RNA Viruses in a Drosophila Schneider 2 cell line (Wu et al. 2010).

11.7.2.2 Endogenous Viruses

Endogenous, i.e. genetically transmitted viruses, require special consideration. Most attention is directed at retroviruses and errantiviruses, their counterparts in insect cells. However, it is important to consider other viruses that may be endogenous. It is now well recognised that about 1 % of children have congenital *Human herpesvirus 6* (HHV-6) infections and 86 % of these are the result of germline transmission of chromosomally integrated virus (Hall et al. 2004; Leong et al. 2007; Hall et al. 2008).

An essentially full length endogenous parvovirus genome has been identified in rats and other species; viral mRNA is expressed from the rat virus although it is defective (Kapoor et al. 2010). Human and other species cells, contain endogenized *Borna disease virus* like sequences that presumably have been reverse transcribed by LINE or other retroelements. (Horie et al. 2010; Belyi et al. 2010). The genomes are defective with N protein integrants predominating. Filovirus sequences are the only other RNA virus sequences that appear to be widely integrated as chromosomal DNA copies (Taylor et al. 2010; Beyli et al. 2010). An interesting question is whether expression of these sequences has been associated with a protective effect against cognate virus infection during evolutionary history.

All vertebrate cells contain endogenous retroviral sequences and they constitute a significant part of the genome, up to 8 % in human cells. Different retroviral species are represented and some of these may be expressed at the mRNA and protein level. In human cells all the endogenous retroviral proviruses are defective

and unable to produce virions capable of infecting other cells but in other species, like cats, infectious endogenous retroviruses can be produced. The feline endogenous virus RD114 has been found as a contaminant of both canine parvovirus seeds grown in feline cells and in vaccines produced, in non-feline cells, from these seeds (Yoshikawa et al. 2010). It is of particular concern that this retrovirus replicates efficiently in canine cells and careful monitoring of the recipients will be required. Retroviruses have contaminated other vaccines in the past including: contamination of, yellow fever, distemper and Marek's disease virus vaccines by avian leukosis virus (Draper 1967; Payne et al. 1966; Zavala and Cheng 2006), babesiosis vaccines by bovine leukemia virus (Rogers et al. 1988) and fowlpox vaccines by reticuloendotheliosis virus (Fadly and Garcia 2006). In addition, endogenous avian retroviral sequences (ALV-E and EAV) may be present in egg or chicken fibroblast produced vaccines although, after extensive evaluation, these are not believed to pose a hazard (Robertson and Minor 1996; Weissmahr et al. 1997; Khan et al. 1998; Shahabuddin et al. 2001; Hussain et al. 2003).

The recent episode involving contaminated parvovirus vaccines highlights the importance of screening adequately for retroviruses. This involves induction studies to initiate the expression of transcriptionally silent viruses and monitoring for their presence by orthogonal methods (Khan et al. 2009; Onions et al. 2010). MPS of the transcriptome and MPS of the supernatant media is a valuable adjunct in the evaluation of retroviral contamination. In human cells, and other cells where full genomic data is available, the method enables one to exclude exogenous retroviruses, both known and unknown. But the strength of the method is best exemplified for cell lines where genomic information may be limited. Vero cells have been extensively used in the production of vaccines although the genomic data is limited. Vero cells were not thought to express a retrovirus but application of MPS and other approaches resulted in the surprising discovery that this widely used and monitored cell line could be induced to express a full length betaretrovirus genome and produce viral particles. (Ma et al. 2011; Onions et al. 2011). MPS was able to show that another retrovirus genome in this cell line, related to baboon endogenous virus, showed no changes in expression on induction and was defective posing no threat to biosafety (Onions et al. 2011). However, it should be noted that even defective retroviruses may be of concern. For instance, an attenuated flavivirus, produced in cells expressing a defective retrovirus, could pseudotype the retrovirus enabling the retroviral genome to infect otherwise non-permissive cells.

11.8 MPS for the Genetic Stability of Bacterial and Viral Vaccines and Viral Vectors

Genetic stability analyses have usually relied on indirect and partial information like PCR of selected regions and restriction enzyme analysis. For bacterial seed stocks it is now routine to sequence the whole genome rather than rely on imprecise

methods. MPS methods produce sequences of thousands of individual viruses within a seed, enabling the detection of variant viruses.

The high mutation rate in RNA viruses can theoretically lead to high levels of variant viruses. This is a critical issue for certain viruses, like oral polio vaccines (OPV), where reversion to neurovirulence can occur. As part of the quality control of OPV, the vaccine is tested for neurovirulence in monkeys or, transgenic mice, as well as by PCR and restriction enzyme analysis for known mutations affecting neurovirulence. Neverov and Chumakov (2010) have shown that MPS can effectively replace the standard molecular methods and additional information is provided like the rapid outgrowth of a capsid mutant when the seed is propagated in cells. In their studies they demonstrated a 0.05 % mutation frequency in control plasmids and 0.1 % in amplified product from the plasmid but 0.12 % in the rederived virus which increased to 0.197 % on culture in Vero cells. Based on this information they used 0.1 % as the threshold to define valid genotypic variants. In analysing a neurovirulent versus a non-neurovirulent OPV, there was 0.35 % of the virulence-associated 472-C mutant in the lot that passed the neurovirulence test, versus 2.4 % in the lot that failed.

In our own evaluation of virus seed stocks on a Roche 454 GS-FLX/FLX+ we have been able to demonstrate a surprising stability in viruses like reoviruses. Reovirus contains 10 dsRNA genomic segments and coverage across the 10 segments varied from 3,000 to 20,000 fold; consequently, unlike Sanger sequencing, the process identified rare variants required for oncolytic activity (Chakrabarty et al. 2014). MPS has also been applied in clinical settings to identify chromosomal integration sites of retroviral vectors and to monitor the clonal evolution of transduced cells. MPS will also play an important role in evaluating vectors for partial recombinants that can lead to replication competent viruses. Modern adenovirus, lentivirus and gammaretrovirus vector systems are far less likely to generate replication competent viruses then their first and second generation counterparts. However, the methods for detecting replication competent virus (RCV) require the use of high volumes of vector material and the sensitivity of detection of RCV can be reduced by the presence of high titre vector. Before replication competent virus is generated there are usually partial non-replication recombinants generated. This is particularly evident for retroviruses because of their diploid genome and capacity for strand switching during reverse transcription. MPS affords a method of detecting these partial recombinants before the generation of RCV and provides additional data on the genetic stability of the vector system.

11.9 Conclusion

As indicated in the recent revision to the WHO guidance on cell substrates (Knezevic et al. 2010), MPS is going to play an increasing part in the safety evaluation of cell substrates and vaccines. While in some cases it will replace older molecular methods it will remain part of a comprehensive panel of orthogonal

assays that include traditional infectivity assay systems (McClenahan et al. 2011; Kolman 2011).

MPS systems are amenable to GMP validation although this is a complex and requires a module by module validation as well a total system validation. However, the power of the technology has already been shown in the discovery of new viruses in raw materials, the finding of novel viruses in insect and primate cell substrates and the discovery of an unexpected contaminant in rotavirus vaccines.

References

Allander T, Emerson SU, Engle RE, Purcell RH, Bukh J (2001) A virus discovery method incorporating DNase treatment and its application to the identification of two bovine parvovirus species. Proc Natl Acad Sci USA 98(20):11609–11614 (Epub 2001 Sept 18)

Allen TM, Altfeld M, Geer SC, Kalife ET, Moore C et al (2005) Selective escape from CD8+ T-cell responses represents a major driving force of human immunodeficiency virus type 1 (HIV-1) sequence diversity and reveals constraints on HIV-1 evolution. J Virol 79:13239–13249

Almeida JL, Hill CR, Cole KD (2011) Authentication of African green monkey cell lines using human short tandem repeat markers. BMC Biotechnol 7(11):102

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Archer J, Baillie G, Watson SJ, Kellam P, Rambaut A, Robertson DL (2012) Analysis of high-depth sequence data for studying viral diversity: a comparison of next generation sequencing platforms using segminator II. BMC Bioinform 23(13):47

Astrovskaya I, Tork B, Mangul S, Westbrooks K, Măndoiu I, Balfe P, Zelikovsky A (2011) Inferring viral quasispecies spectra from 454 pyrosequencing reads. BMC Bioinform 12(Suppl 6):S1 (Epub 2011 July 28)

Bar KJ, Tsao CY, Iyer SS, Decker JM, Yang Y, Bonsignori M, Chen X, Hwang KK, Montefiori DC, Liao HX, Hraber P, Fischer W, Li H, Wang S, Sterrett S, Keele BF, Ganusov VV, Perelson AS, Korber BT, Georgiev I, McLellan JS, Pavlicek JW, Gao F, Haynes BF, Hahn BH, Kwong PD, Shaw GM (2012) Early low-titer neutralizing antibodies impede HIV-1 replication and select for virus escape. PLoS Pathog 8(5):e1002721 (Epub 2012 May 31)

Belyi VA, Levine AJ, Skalka AM (2010) Unexpected inheritance: multiple integrations of ancient Bornavirus and Ebolavirus/Marburgvirus sequences in vertebrate genomes. PLoS Pathog 6:1001030

Biebricher CK, Eigen M (2006) What is a quasispecies? Curr Top Microbiol Immunol 99:1–31 (Review)

Borrow P, Lewicki H, Wei XP, Horwitz MS, Peffer N et al (1997) Antiviral pressure exerted By HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat Med 3:205–211

Bull RA, Luciani F, McElroy K, Gaudieri S, Pham ST, Chopra A, Cameron B, Maher L, Dore GJ, White PA, Lloyd AR (2011) Sequential bottlenecks drive viral evolution in early acute hepatitis C virus infection. PLoS Pathog 7(9):e1002243 (Epub 2011 Sept 1)

Chakrabarty R, Tran H, Fortin Y, Yu Z, Shen SH, Kolman J, Onions D, Voyer R, Hagerman A, Serl S, Kamen A, Thompson B, Coffey M (2014) Evaluation of homogeneity and genetic stability of REOLYSIN (pelareorep) by complete genome sequencing of reovirus after large scale production. Appl Microbiol Biotechnol 98(4):1763–1770. doi:10.1007/s00253-013-5499-0 (Epub 2014 Jan 14)

Cordey S, Junier T, Gerlach D, Gobbini F, Farinelli L, Zdobnov EM, Winther B, Tapparel C, Kaiser L (2010) Rhinovirus genome evolution during experimental human infection. PLoS ONE 5(5):10588

de Vries M, Deijs M, Canuti M, van Schaik BD, Faria NR, van de Garde MD, Jachimowski LC, Jebbink MF, Jakobs M, Luyf AC, Coenjaerts FE, Claas EC, Molenkamp R, Koekkoek SM, Lammens C, Leus F, Goossens H, Ieven M, Baas F, van der Hoek L (2011) A sensitive assay for virus discovery in respiratory clinical samples. PLoS ONE 6(1):16118

Domingo E, Sabo D, Taniguchi T, Weissmann C (1978) Nucleotide sequence heterogeneity of an RNA phage population. Cell 13:735–744

Domingo E, Martin V, Perales C, Grande-Pérez A, García-Arriaza J, Arias A (2006) Viruses as quasispecies: biological implications. Curr Top Microbiol Immunol 299:51–82 (Review)

Draper CC (1967) A yellow fever vaccine free from avian leucosis viruses. J Hyg (Lond) 65 (4):505–513

Eigen M, Schuster P (1977) The hypercycle. A principle of natural self-organization. Part A: emergence of the hypercycle. Naturwissenschaften 64:541–565

Eltonsy N, Gabisi V, Li X, Russe KB, Mills GB, Stemke-Hale K (2012) Detection algorithm for the validation of human cell lines. Int J Cancer 131(6):E1024–E1030. doi:10.1002/ijc.27533 (Epub 2012 Apr 12)

Eriksson N, Pachter L, Mitsuya Y, Rhee SY, Wang C, Gharizadeh B, Ronaghi M, Shafer RW, Beerenwinkel N (2008) Viral population estimation using pyrosequencing. PLoS Comput Biol 4(4):e1000074

Fadly A, Garcia MC (2006) Detection of reticuloendotheliosis virus in live virus vaccines of poultry. Dev Biol (Basel) 126:301–305

Feng H, Shuda M, Chang Y, Moore PS (2008) Clonal integration of a polyomavirus in 394 human Merkel cell carcinoma. Science 319:1096–1100

Fischer W, Ganusov VV, Giorgi EE, Hraber PT, Keele BF, Leitner T, Han CS, Gleasner CD, Green L, Lo CC, Nag A, Wallstrom TC, Wang S, McMichael AJ, Haynes BF, Hahn BH, Perelson AS, Borrow P, Shaw GM, Bhattacharya T, Korber BT (2010) Transmission of single HIV-1 genomes and dynamics of early immune escape revealed by ultra-deep sequencing. PLoS ONE 5(8):12303

Fonseca-Coronado S, Escobar-Gutiérrez A, Ruiz-Tovar K, Cruz-Rivera MY, Rivera-Osorio P, Vazquez-Pichardo M, Carpio-Pedroza JC, Ruíz-Pacheco JA, Cazares F, Vaughan G (2012) Specific detection of naturally occurring hepatitis C virus mutants with resistance to telaprevir and boceprevir (protease inhibitors) among treatment-naïve infected individuals. J Clin Microbiol 50(2):281–287 (Epub 2011 Nov 23)

Frensing T, Seitz C, Heynisch B, Patzina C, Kochs G, Reichl U (2011) Efficient influenza B virus propagation due to deficient interferon-induced antiviral activity in MDCK cells. Vaccine 29 (41):7125–7129 (Epub 2011 June 7)

Ghedin E, Laplante J, DePasse J, Wentworth DE, Santos RP, Lepow ML, Porter J, Stellrecht K, Lin X, Operario D, Griesemer S, Fitch A, Halpin RA, Stockwell TB, Spiro DJ, Holmes EC, St George K (2011) Deep sequencing reveals mixed infection with 2009 pandemic influenza A (H1N1) virus strains and the emergence of oseltamivir resistance. J Infect Dis 203(2):168–174

Hall C, Caserta M, Schnabel K et al (2004) Congenital infections with human herpesviruses 6 and 7. J Pediatr 145:472–477

Hall C, Caserta M, Schnabel K et al (2008) Chromosomal integration of human herpesvirus 6 is the major mode of congenital human herpesvirus 6 infection. Pediatrics 122:513–520

Harismendy O, Ng PC, Strausberg RL, Wang X, Stockwell TB, Beeson KY, Schork NJ, Murray SS, Topol EJ, Levy S, Frazer KA (2009) Evaluation of next generation sequencing platforms for population targeted sequencing studies. Genome Biol 10(3):R32

Haynes BF, Hahn BH, Kwong PD, Shaw GM (2012) Early low-titer neutralizing antibodies impede HIV-1 replication and select for virus escape. PLoS Pathog 8(5):e1002721 (Epub 2012 May 31)

Henn MR, Boutwell CL, Charlebois P, Lennon NJ, Power KA, Macalalad AR, Berlin AM, Malboeuf CM, Ryan EM, Gnerre S, Zody MC, Erlich RL, Green LM, Berical A, Wang Y,

Casali M, Streeck H, Bloom AK, Dudek T, Tully D, Newman R, Axten KL, Gladden AD, Battis L, Kemper M, Zeng Q, Shea TP, Gujja S, Zedlack C, Gasser O, Brander C, Hess C, Günthard HF, Brumme ZL, Brumme CJ, Bazner S, Rychert J, Tinsley JP, Mayer KH, Rosenberg E, Pereyra F, Levin JZ, Young SK, Jessen H, Altfeld M, Birren BW, Walker BD, Allen TM (2012) Whole genome deep sequencing of HIV-1 reveals the impact of early minor variants upon immune recognition during acute infection. PLoS Pathog 8(3):e1002529 (Epub 2012 Mar 8)

Holmes EC (2010) The RNA virus quasispecies: fact or fiction? J Mol Biol 400(3):271–273 (Epub 2010 May 20. Review)

Hori M, Honda T, Suzuki Y, Kobayashi Y, Daito T, Oshida T, Ikuta K, Jern P, Gojobori T, Coffin JM, Tomonaga K (2010) Endogenous non-retroviral RNA virus elements in mammalian genomes. Nature 463(7277):84–87

Höper D, Kalthoff D, Hoffmann B, Beer M (2012) Highly pathogenic avian influenza virus subtype H5N1 escaping neutralization: more than HA variation. J Virol 86(3):1394–1404 (Epub 2011 Nov 16)

Hussain AI, Johnson JA, Da Silva Freire M, Heneine W (2003) Identification and characterization of avian retroviruses in chicken embryo-derived yellow fever vaccines: investigation of transmission to vaccine recipients. J Virol 77(2):1105–1111

ICH Q8(R2) Pharmaceutical Development (2009) U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Biologics Evaluation and Research (CBER). November 2009 ICH. Revision

Jabara CB, Jones CD, Roach J, Anderson JA, Swanstrom R (2011) Accurate sampling and deep sequencing of the HIV-1 protease gene using a Primer ID. Proc Natl Acad Sci USA 108 (50):20166–20171 (Epub 2011 Nov 30)

Kapoor A, Simmonds P, Lipkin WI (2010) Discovery and characterization of mammalian endogenous parvoviruses. J Virol 84(24):12628–12635. doi:10.1128/JVI.01732-10 (Published online 2010 Oct 13)

Kapoor A, Simmonds P, Dubovi EJ, Qaisar N, Henriquez JA, Medina J, Shields S, Lipkin WI (2011) Characterization of a canine homolog of human Aichivirus. Virol 85(21):11520–11525 (Epub 2011 Aug 31)

Khan AS, Maudru T, Thompson A, Muller J, Sears JF, Peden KW (1998) The reverse transcriptase activity in cell-free medium of chicken embryo fibroblast cultures is not associated with a replication-competent retrovirus. J Clin Virol 11(1):7–18

Khan AS, Ma W, Ma Y, Kumar A, Williams DK, Muller J, Ma H, Galvin TA (2009) Proposed algorithm to investigate latent and occult viruses in vaccine cell substrates by chemical induction. Biologicals 37(3):196–201

Klug A (2010) The discovery of zinc fingers and their development for practical applications in gene regulation and genome manipulation. Q Rev Biophys 43(1):1–21 (Epub 2010)

Knezevic I, Stacey G, Petricciani J, Sheets R (2010) WHO study group on cell substrates. Evaluation of cell substrates for the production of biologicals: revision of WHO recommendations. Report of the WHO study group on cell substrates for the production of biologicals, 22–23 April 2009, Bethesda, USA. Biologicals 38(1):162–169 (Epub 2009 Oct 8. Review)

Kolman JL (2011) Massively parallel sequencing for the detection of adventitious viruses. PDA J Pharm Sci Technol 65(6):663–667

Leong HN, Tuke PW, Tedder RS, Khanom AB, Eglin RP et al (2007) The prevalence of chromosomally integrated human herpesvirus 6 genomes in the blood of UK blood donors. J Med Virol 79:45–51. doi:10.1002/jmv.2076

Li L, Kapoor A, Slikas B, Bamidele OS, Wang C, Shaukat S, Masroor MA, Wilson ML, Ndjango JB, Peeters M, Gross-Camp ND, Muller MN, Hahn BH, Wolfe ND, Triki H, Bartkus J, Zaidi SZ, Delwart E (2010) Multiple diverse circoviruses infect farm animals and are commonly found in human and chimpanzee feces. J Virol 84(4):1674–1682 (Epub 2009 Dec 9)

Li L, Pesavento PA, Shan T, Leutenegger CM, Wang C, Delwart E (2011a) Viruses in diarrhoeic dogs include novel kobuviruses and sapoviruses. J Gen Virol 92(Pt 11):2534–2541 (Epub 2011a July 20)

Li L, Shan T, Wang C, Côté C, Kolman J, Onions D, Gulland FM, Delwart E (2011b) The fecal viral flora of California sea lions. J Virol 85(19):9909–9917 (Epub 2011 July 27)

Lloyd S, Mead S, Collinge J (2011) Genetics of prion disease. Top Curr Chem 305:1–22 (Review)

Ma H, Ma Y, Ma W, Williams DK, Galvin TA, Khan AS (2011) Chemical induction of endogenous retrovirus particles from the vero cell line of African green monkeys. J Virol 85 (13):6579–6588 (Epub 2011 May 4)

Ma H, Galvin TA, Glasner DR, Shaheduzzaman S, Khan AS (2014) Identification of a novel rhabdovirus in spodoptera frugiperda cell lines. J Virol 88(12):6576–6585 (Epub 2014 Mar 2)

Macalalad AR, Zody MC, Charlebois P, Lennon NJ, Newman RM, Malboeuf CM, Ryan EM, Boutwell CL, Power KA, Brackney DE, Pesko KN, Levin JZ, Ebel GD, Allen TM, Birren BW, Henn MR (2012) Highly sensitive and specific detection of rare variants in mixed viral populations from massively parallel sequence data. PLoS Comput Biol 8(3):e1002417 (Epub 2012 Mar 15)

McClenahan S, Uhlenhaut C, Krause PR (2011) Regulatory approaches for control of viral contamination of vaccines PDA J Pharm Sci Technol 65(6):557–562, 663

Meng C, Zhou Z, Jiang K, Yu S, Jia L, Wu Y, Liu Y, Meng S, Ding C (2012) Newcastle disease virus triggers autophagy in U251 glioma cells to enhance virus replication. Arch Virol 157 (6):1011

Metzker ML (2010) Sequencing technologies—the next generation. Nat Rev Genet 11(1):31–46

Moore RA, Warren RL, Freeman JD, Gustavsen JA, Chénard C, Friedman JM, Suttle CA, Zhao Y, Holt RA (2011) The sensitivity of massively parallel sequencing for detecting candidate infectious agents associated with human tissue. PLoS ONE 6(5):e19838 (Epub 2011 May 13)

Mosca JD, Pitha PM (1986) Transcriptional and posttranscriptional regulation of exogenous human beta interferon gene in simian cells defective in interferon synthesis. Mol Cell Biol 6 (6):2279–2283

Nakamura T, Asano A, Okano S, Ko JH, Kon Y, Watanabe T, Agui T (2005) Intracellular localization and antiviral property of canine Mx proteins. J Interferon Cytokine Res 25 (3):169–173

Nardone RM (2008) Curbing rampant cross-contamination and misidentification of cell lines. BioTechniques 45(3):221

Neverov A, Chumakov K (2010) Massively parallel sequencing for monitoring genetic consistency and quality control of live viral vaccines. Proc Natl Acad Sci USA 107 (46):20063–20068 (Epub 2010 Nov 1)

Nims Raymond W, Dusing Sandra K, Wang-Ting H, Lovatt A, Reid CG, Onions D, Milne EW (2008) Detection of cache valley virus in biologics manufactured in CHO cells. BioPharm Int 21(10):89

Onions D (2004) Animal virus contaminants of biotechnology products. Dev Biol (Basel) 118:155–163

Onions D, Egan W, Jarrett R, Novicki D, Gregersen JP (2010) Validation of the safety of MDCK cells as a substrate for the production of a cell-derived influenza vaccine. Biologicals 38 (5):544–551

Onions D, Kolman J (2010) Massively parallel sequencing, a new method for detecting adventitious agents. Biologicals 38(3):377–380 (Epub 2010 Mar 24)

Onions D (2011) Overview of emerging technologies to detect adventitious agents. PDA J Pharm Sci Technol 65(6):654–659

Onions D, Côté C, Love B, Toms B, Koduri S, Armstrong A, Chang A, Kolman J (2011) Ensuring the safety of vaccine cell substrates by massively parallel sequencing of the transcriptome. Vaccine 29(41):7117–7121 (Epub 2011 June 7)

Palacios G, Druce J, Du L, Tran T, Birch C, Briese T, Conlan S, Quan PL, Hui J, Marshall J, Simons JF, Egholm M, Paddock CD, Shieh WJ, Goldsmith CS, Zaki SR, Catton M, Lipkin WI (2008) A new arenavirus in a cluster of fatal transplant-associated diseases. N Engl J Med 358 (10):988–989

Parameswaran P, Charlebois P, Tellez Y, Nunez A, Ryan EM, Malboeuf CM, Levin JZ, Lennon NJ, Balmaseda A, Harris E, Henn MR (2012) Genome-wide patterns of intrahuman dengue

virus diversity reveal associations with viral phylogenetic clade and interhost diversity. J Virol 86(16):8546–8558 (Epub 2012 May 30)
Payne LN, Biggs PM, Chubb RC, Bowden RS (1966) Contamination of egg-adapted canine distemper vaccine by avian leukosis virus. Vet Rec 78(2):45–48
Phan TG, Vo NP, Bonkoungou IJ, Kapoor A, Barro N, O'Ryan M, Kapusinszky B, Wang C, Delwart E (2012) Acute diarrhea in West-African children: diverse enteric viruses and a novel parvovirus genus. J Virol (Epub ahead of print)
Plavsic and Bolin (2001) Resistence of porcine circovirus to gamma irradiation. Biopharm Int 14:32–36
Price DA, Goulder PJ, Klenerman P, Sewell AK, Easterbrook PJ et al (1997) Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. Proc Natl Acad Sci USA 94:1890–1895
Prosperi MC, Salemi M (2012) QuRe: software for viral quasispecies reconstruction from next-generation sequencing data. Bioinformatics 28(1):132–133 (Epub 2011 Nov 15)
Quince C, Lanzen A, Davenport RJ, Turnbaugh PJ (2011) Removing noise from pyrosequenced amplicons. BMC Bioinform 28(12):38
Richman DD, Wrin T, Little SJ, Petropoulos CJ (2003) Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc Natl Acad Sci USA 100:4144–4149
Robertson JS, Minor P (1996) Reverse transcriptase activity in vaccines derived from chick cells. Biologicals 24(3):289–290
Rogers RJ, Dimmock CK, de Vos AJ, Rodwell BJ (1988) Bovine leucosis virus contamination of a vaccine produced in vivo against bovine babesiosis and anaplasmosis. Aust Vet J 65(9):285
Russell CA, Fonville JM, Brown AE, Burke DF, Smith DL, James SL, Herfst S, van Boheemen S, Linster M, Schrauwen EJ, Katzelnick L, Mosterín A, Kuiken T, Maher E, Neumann G, Osterhaus AD, Kawaoka Y, Fouchier RA, Smith DJ (2012) The potential for respiratory droplet-transmissible A/H5N1 influenza virus to evolve in a mammalian host. Science 336 (6088):1541–1547
Salmela L, Schröder J (2011) Correcting errors in short reads by multiple alignments. Bioinformatics 27(11):1455–1461 (Epub 2011 Apr 5)
Sanjuán R, Nebot MR, Chirico N, Mansky LM, Belshaw R (2010) Viral mutation rates. J Virol 84 (19):9733–9748. doi:10.1128/JVI.00694-10 (Epub 2010 July 21. Review)
Seitz C, Isken B, Heynisch B, Rettkowski M, Frensing T, Reichl U (2012) Trypsin promotes efficient influenza vaccine production in MDCK cells by interfering with the antiviral host response. Appl Microbiol Biotechnol 93(2):601–611 (Epub 2011 Sept 14)
Shahabuddin M, Sears JF, Khan AS (2001) No evidence of infectious retroviruses in measles virus vaccines produced in chicken embryo cell cultures. J Clin Microbiol 39(2):675–684
Siebrasse EA, Reyes A, Lim ES, Zhao G, Mkakosya RS, Manary MJ, Gordon JI, Wang D (2012) Identification of MW polyomavirus, a novel polyomavirus in human stool. J Virol (Epub ahead of print)
Skums P, Dimitrova Z, Campo DS, Vaughan G, Rossi L, Forbi JC, Yokosawa J, Zelikovsky A, Khudyakov Y (2012) Efficient error correction for next-generation sequencing of viral amplicons. BMC Bioinform 13(10):6
Smith JL, Grey FE, Uhrlaub JL, Nikolich-Zugich J, Hirsch AJ (2012) West Nile virus induction of the cellular microRNA, Hs_154, contributes to viral-mediated apoptosis through repression of anti-apoptotic factors. J Virol (Epub ahead of print)
Sullivan R, Fassolitis AC, Larkin EP, Read RB Jr (1971) Peeler JT inactivation of thirty viruses by gamma radiation. Appl Microbiol 22(1):61–65
Svarovskaia ES, Martin R, McHutchison JG, Miller MD, Mo H (2012) Abundant drug-resistant NS3 mutants detected by deep sequencing in HCV-infected patients undergoing NS3 protease inhibitor monotherapy. J Clin Microbiol (Epub ahead of print)
Tapparel C, Cordey S, Junier T, Farinelli L, Van Belle S, Soccal PM, Aubert JD, Zdobnov E, Kaiser L (2011) Rhinovirus genome variation during chronic upper and lower respiratory tract infections. PLoS ONE 6(6):e21163 (Epub 2011 June 21)

Taylor DJ, Leach RW, Bruenn J (2010) Filoviruses are ancient and integrated into mammalian genomes. BMC Evol Biol 10:193

Tsibris AM, Korber B, Arnaout R, Russ C, Lo CC, Leitner T, Gaschen B, Theiler J, Paredes R, Su Z, Hughes MD, Gulick RM, Greaves W, Coakley E, Flexner C, Nusbaum C, Kuritzkes DR (2009) Quantitative deep sequencing reveals dynamic HIV-1 escape and large population shifts during CCR5 antagonist therapy in vivo. PLoS ONE 4(5):e5683

Verbinnen T, Van Marck H, Vandenbroucke I, Vijgen L, Claes M, Lin TI, Simmen K, Neyts J, Fanning G, Lenz O (2010) Tracking the evolution of multiple in vitro hepatitis C virus replicon variants under protease inhibitor selection pressure by 454 deep sequencing. J Virol 84 (21):11124–11133 (Epub 2010 Aug 25)

Vester D, Rapp E, Kluge S, Genzel Y, Reichl U (2010) Virus-host cell interactions in vaccine production cell lines infected with different human influenza A virus variants: a proteomic approach. J Proteomics 73(9):1656–1669 (Epub 2010 May 10)

Victoria JG, Wang C, Jones MS, Jaing C, McLoughlin K, Gardner S, Delwart EL (2010) Viral nucleic acids in live-attenuated vaccines: detection of minority variants and an adventitious virus. J Virol 84(12):6033–6040 (Epub 2010 Apr 7)

Wang XH, Aliyari R, Li WX, Li HW, Kim K, Carthew R, Atkinson P, Ding SW (2006) RNA interference directs innate immunity against viruses in adult Drosophila. Science 312 (5772):452–454 (Epub 2006 Mar 23)

Wang C, Mitsuya Y, Gharizadeh B, Ronaghi M, Shafer RW (2007) Characterization of mutation spectra with ultra-deep pyrosequencing: application to HIV-1 drug resistance. Genome Res 17 (8):1195–1201 (Epub 2007 June 28)

Weissmahr RN, Schüpbach J, Böni J (1997) Reverse transcriptase activity in chicken embryo fibroblast culture supernatants is associated with particles containing endogenous avian retrovirus EAV-0 RNA. J Virol 71(4):3005–3012

Whiteford N, Haslam N, Weber G, Prügel-Bennett A, Essex JW, Roach PL, Bradley M, Neylon C (2005) An analysis of the feasibility of short read sequencing. An analysis of the feasibility of short read sequencing. Nucleic Acids Res 33(19):171

Wu Q, Luo Y, Lu R, Lau N, Lai EC, Li WX, Ding SW (2010) Virus discovery by deep sequencing and assembly of virus-derived small silencing RNAs. Proc Natl Acad Sci USA 107 (4):1606–1611 (Epub 2010 Jan 4)

Yoshikawa R, Sato E, Miyazawa T (2011) Contamination of infectious RD-114 virus in vaccines produced using non-feline cell lines. Biologicals 39(1):33–37 (Epub 2010 Dec 8)

Yozwiak NL, Skewes-Cox P, Stenglein MD, Balmaseda A, Harris E, DeRisi JL (2012) Virus identification in unknown tropical febrile illness cases using deep sequencing. PLoS Negl Trop Dis 6(2):e1485 (Epub 2012 Feb 7)

Zagordi O, Geyrhofer L, Roth V, Beerenwinkel N (2010) Deep sequencing of a genetically heterogeneous sample: local haplotype reconstruction and read error correction. J Comput Biol 17(3):417–428

Zavala G, Cheng S (2006) Detection and characterization of avian leukosis virus in Marek's disease vaccines. Avian Dis 50(2):209–215

Chapter 12
Quality-by-Design: As Related to Analytical Concepts, Control and Qualification

Beth Junker, Earl Zablackis, Thorsten Verch, Tim Schofield and Pierre Douette

12.1 Quality-by-Design for Analytical Methods

12.1.1 Roots of QbD

In 2002, the US Food and Drug Administration (FDA) outlined in its guidance "Pharmaceutical CGMPs for the twenty-first Century: A Risk-Based Approach" a new science- and risk-based approach that encourages manufacturers to develop robust processes and appropriate control strategies thus supporting continuous improvement and product quality (Catania 2011). Over the years this approach has evolved as the pharmaceutical industry and regulatory authorities have distilled down different standards of practice already in place for decades in other industries to a core concept called Quality-by-Design (QbD). This has also given rise to a

All authors contributed equally to this chapter.

B. Junker (✉)
Kenilworth, New Jersey, USA
e-mail: Beth.junker@merck.com

E. Zablackis
Swiftwater, PA, USA
e-mail: Earl.Zablackis@sanofipasteur.com

T. Verch
West Point, PA, USA
e-mail: thorsten_verch@merck.com

T. Schofield
MedImmune, Gaithersburg, Maryland, USA
e-mail: timothylschofield@gmail.com

P. Douette
BioCompliance Services, Avenue du Luxembourg 58, 4020 Liège, Belgium

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6_12

significant body of novel regulatory documents introducing the terminology and discussing operational implications fostered by the QbD initiative: International Conference on Harmonization (ICH) Q8 and Q11 on pharmaceutical and API development, Q9 on quality risk management, Q10 on quality systems and FDA Process Analytical Technology and FDA Process Validation (Guidance for Industry 2006, 2009, 2011; ICH 2012; ICH 2009; CDER/CVM/ORA 2004).

The ICH Q8(R2) guideline describes QbD as a "systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management." This document sets the foundation for QbD where key components that link product and process—such as target product profile, critical quality attributes, and design space—are presented as the way to operate processes within defined variation which ensures product quality and patient safety. From a current regulatory standpoint, a systematic approach as proposed through QbD represents the desired state expected by regulators for Chemistry, Manufacturing, and Control (CMC). It ensures the manufacturing process can consistently deliver product quality in commercial operations, even if QbD submission is not a regulatory requirement. Among regulatory incentives for adopting QbD, special highlight is given to the potential for regulatory flexibility—principally toward post-approval change management—enabled by a good understanding of what sources of process variation are acceptable for keeping the product safe (Chen 2007). It is then not surprising that, in its 2011 strategic plan for regulatory science (Critical Path Initiative), the US FDA has made the promotion of QbD principles one of its Top 10 priorities to support new approaches in manufacturing process development (FDA 2004).

Although implementation of QbD within the industry historically has been a slow process, the approach is now widely acknowledged as providing the desired development path that matches scientific understanding and risk-based compliance for pharmaceutical development. With the approach being further translated into concrete theoretical case studies (best illustrated by the A-MAb and A-Vax initiatives (CMC Biotech Working Group 2009; CMC Vaccine Working Group 2010), the industry also recognizes the potentially significant business benefits arising from adoption of a QbD-compliant process from the time of early development. These benefits include shorter time to market; reliable manufacturing operations supported by continuous improvement, and reduced cost and/or regulatory hurdles related to inconsistent product quality. Finally implementing a structured science- and risk-based development path also has the benefit of improving interactions with the regulators by matching their current expectations.

QbD for analytical methods parallels QbD for manufacturing processes. In principle an analytical method can be regarded as a process. The product of the analytical method process is a measurement which is used alone or in aggregate with other measurements to make a decision. The customer for the product is the decision maker who depends upon the reliability of the method for their decision making. Many of the concepts associated with QbD for the manufacturing process can be mapped to similar concepts in the analytical method process. This is illustrated in Table 12.1.

Table 12.1 Comparison of process and analytical QbD

Process concept	Analytical counterpart
Target Product Profile (TPP)	Analytical Target profile (ATP)
• Target clinical performance, manufacturing, and commercial requirements	• Target analytical performance, testing laboratory, and customer requirements
Critical Quality Attributes(CQAs)	Critical Method Attributes (CMAs)
• Potency	• Precision
• Aggregation	• Sensitivity
• Purity	• Accuracy
Specifications (acceptance criteria)	Acceptance criteria
• 80–125 % potency	• %GCV < 10 %
• Purity > 95 %	• LLOQ > 1 ng/mL
Critical process parameters (CPPs)	Critical method parameters (CMPs)
• pH, time, temperature	• pH, time, temperature
Process control strategy	Method control strategy
• Comparability protocols	• Method comparability or bridging protocols
• Process technology transfer	• Method transfer
Continuous process verification	Analytical method maintenance
• Continuous review and updating of process knowledge	• Continuous review and updating of analytical knowledge

LLOQ lower limit of quantification; *GCV* geometric coefficient of variation

In this regard the thinking and the tools which are useful in QbD for manufacturing process development are equally useful for analytical method development. For example, the control strategy supports successful transfers for either processes or analytical methods.

One consideration in applying QbD to methods is the effort to execute the framework for several dozen methods, each with several procedural steps, might in the aggregate be much more significant than for a single manufacturing process. Although differences between QbD for drug products and QbD for analytical methods obviously exist, many of the tools are similar as is the objective of upfront designed quality working toward patient's benefits.

12.1.2 Objectives and Benefits of Applying QbD to Analytical Methods

The QbD approach intends to build quality into a process/assay rather than test it in through extensive qualification and validation. The result is consistent with controlled assay performance within predefined boundaries that ensure predetermined quality expectations are met. As mentioned in Sect. 12.1.1, regulatory quality expectations are the main driver behind QbD since reliable analytical performance is a key aspect of product quality and safety ensuring release of acceptable product.

Method reliability including measured results is linked to critical method attributes (CMAs) affecting method capability, for example, accuracy, precision, or specificity (Guidance for Industry 2009). QbD for analytical methods systematically investigates and controls these parameters leading to an increased knowledge space around method parameters and reduced variability by controlling assay conditions and risks.

Beyond alignment of analytical method performance to regulatory quality expectations, QbD directly impacts business aspects through reducing variability which translates into improved analytical quality. This includes potentially decreased data variability (greater method precision) as well as greater performance consistency (fewer method invalid and out-of-trend results), with positive down-stream effects on quality management and ultimately costs. When incorporating knowledge management into the QbD process, long-term life-cycle management throughout method transfers and comparability protocols, personnel changes, and method improvements is facilitated as well. In the long-run, it may become acceptable to make changes within the design space of a method with only limited validation and regulatory review.

The systematic approach of QbD combined with sound science helps in achieving these objectives by identifying, mitigating and controlling method risks, as well as by thorough examination of the method design space with respect to required method performance to meet quality and business (i.e., performance) targets. Although this may sound straightforward and obvious at first hand, a detailed look quickly unveils the complexity of true QbD. We will examine some of these aspects within this chapter.

12.1.3 Framework for Quality-by-Design for Analytical Methods

The overarching QbD objective can be achieved by systematically addressing quality aspects at several stages of the method life-cycle: Development, Transfers, Daily Operations, and Continuous Maintenance/Improvement. Knowledge Management of method understanding acquired throughout this process is a key component tying individual steps together (Fig. 12.1 and Sect. 12.3).

Method development is initiated by predefining quality and business performance requirements using an Analytical Target Profile (ATP, Sect. 12.1.4.1). Rather than developing a method solely based on technical feasibility and thereby potentially over- or underachieving objectives (traditional approach), QbD-based development is targeted specifically to the purpose of the method. However, often method development starts concurrently with process development so that exact method requirements may not have been defined yet. In these cases, it is helpful to refer to the (Quality) Target Product Profile ((Q)TPP) to develop agreed upon assumed process targets. Although the final process may differ, these assumed targets could be used as surrogates to derive preliminary method requirements that are updated as more information becomes available.

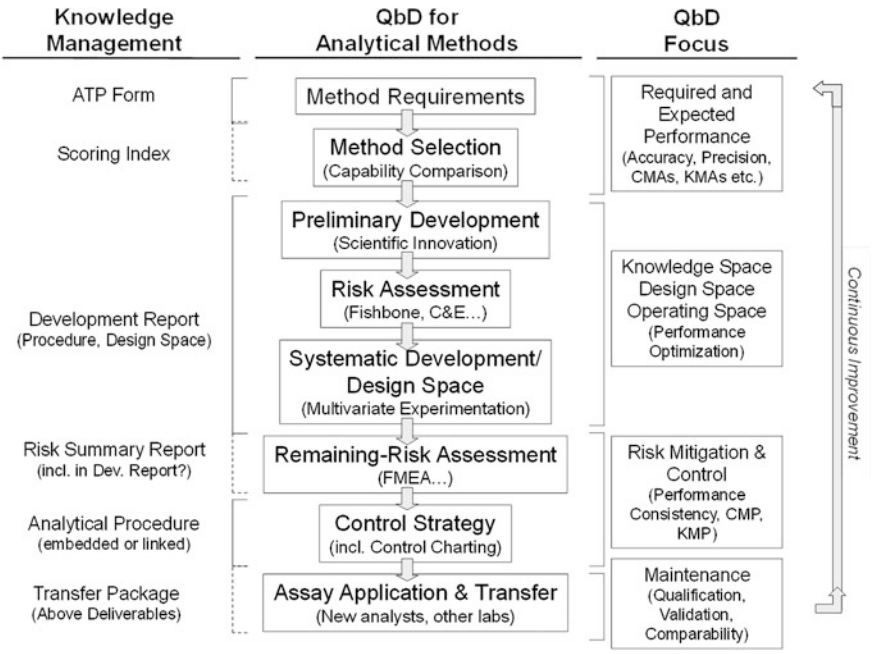


Fig. 12.1 Schematic process for QbD of analytical methods (*ATP* Analytical Target Profile, *CMA* Critical Method Attribute (e.g., accuracy, precision), *CMP* Critical Method Parameter, *KMP* Key Method Parameter, *C&E* Cause and Effects Matrix, *DoE* Design of Experiments, *KMA* Key Method Attributes (e.g. throughput)

After establishing an ATP, QbD includes systematic development at all steps starting at method selection based on evaluation of the capability of potential method candidates to meet ATP requirements. This selection process should not only consider preliminary data but also other quality and business aspects such as transferability or risks (Sect. 12.1.4.2). Systematic risk assessment tools (Sect. 12.1.4.3) are used to identify method factors that potentially can affect method performance. Boundaries and impacts of identified high-risk factors can then be assessed using unifactorial and multifactorial statistical Design of Experiments (DoE, Sect. 12.1.4.4) and/or mitigated using a Control Strategy (Sect. 12.1.4.5).

DoE tools in particular help define a multidimensional design space beyond traditional ranges set for individual method factors around normal assay performance. In contrast to one-factor/variable-at-a-time (OFAT/OVAT) experimentation, DoE varies several experimental factors together allowing the potential identification of synergistic interactions as well as defining system suitability limits. This allows not only to optimize the method within the design space toward the predefined requirements but also to potentially change it within this design space. Thorough understanding of all controlled and uncontrolled aspects affecting quality and business performance is the foundation to reduce variability during daily operations.

Although targeted method optimization goes a long way, some critical method parameters may be incorporated into the control strategy (Sect. 12.1.4.5) that is rooted in systematic risk assessment. Critical and key method parameters, identified during risk assessment, and understood during method development, may be controlled experimentally during daily operations (e.g., temperature), or some may require processes put in place for long-term method maintenance (e.g., assay reagents).

In the QbD framework, the entire body of knowledge, from the analytical target profile to development data and from risk assessment to final control strategies, becomes part of the analytical method. Additional knowledge acquired over time during method use is added to form the basis for continuous improvement (Sect. 12.3).

A number of tools are available and discussed in the following paragraphs to aid with systematic implementation of a QbD framework for bioanalytical methods. When using these tools, three essential aspects of QbD for Analytical Methods should be kept in mind:

(1) QbD is not a check-box exercise! Although the tools are a significant help to focus and align thinking, their superficial execution will not help in achieving the desired quality and business goals. Sound science remains at the core of bioanalytical methods, and findings obtained using statistical and other QbD tools need to be reviewed based on scientific experience and prior knowledge.
(2) Take a holistic view of quality of analytical methods to include all method factors that might influence the quality and business performance. Often, scientists focus on obvious primary experimental effects ignoring or downplaying seemingly secondary effects, such as ensuring performance consistency by well-written and well-presented analytical procedures or by reducing complexities. Frequently, effects attributed to analysts can be viewed as a method execution error and, more often than not, can be prevented by analyzing root-causes appropriately followed by addressing failure modes.
(3) QbD is not a radically new concept. Method development targets, risk assessments, and control strategies have been used to a certain extent for many years in sound bioanalysis even if not specifically named that way. The advantage of QbD is a more systematic and unbiased approach to analytical science through a combination of QbD tools and life-cycle management.

12.1.4 QbD Tools

12.1.4.1 Analytical Target Profile

To apply the concepts of QbD (Guidance for Industry 2009) to analytical methods and their development, the Analytical Target Profile, or ATP, was described by Schweitzer et al. (2010). The ATP is a mechanism for guiding the development and

validation of an analytical method and conceptually, could act as a new mechanism for describing analytical methods in regulatory submissions. Such mechanisms still require alignment with regulatory authorities. The ATP is a tool for systematic development of methods that relates directly to product CQAs. (*NOTE* Not all methods measure a CQA but can be for in-*process control such that they can impact CQA*, *or for product characterization*). The ATP defines what a method measures and within what ranges/limits method characteristics (accuracy, precision, specificity, etc.) must perform. It forms the basis for declaring suitability for intended use (i.e., fit for purpose). The concept of the ATP can also be applied to analytical methods used strictly for characterization of DS, DP, or intermediates. For example, an orthogonal method used to augment or to verify the output of a release method during development should also be scientifically sound and demonstrated to be suitable for its intended use. The use of an ATP allows one to document the performance requirements for the orthogonal method.

The ATP should be initiated early in the analytical method life-cycle and amended as the requirements and constraints on the method and/or manufacturing process evolve. During process development a new product team may request that a method designed for release of DS be applied in upstream process monitoring where the sample matrix may be quite different than that of the released DS. In this case it is possible for the same analytical method to have a different ATP. For example, an upstream sample may have an interfering component such that method is slightly biased leading to different criteria for specificity and accuracy. Key elements of the ATP include: (1) a survey of the uses for the method from development through licensed product control; (2) requirements on performance characteristics that assure method quality and reliable decisions, e.g., meeting Total Error limit (combined accuracy and precision requirement) during routine testing assures confidence in test result reliability for quality disposition; (3) requirements on sample types tested; and (4) practical requirements associated with long-term performance of the method. Method characteristics in the ATP that are directly or indirectly related to measurement quality should be optimized and monitored (see Sect. 12.1.4.6).

Measurement quality is related to the method capability to enable risk-based decision making. Thus, a method which is used to quantify or detect low levels of an impurity or an adventitious agent should provide accurate measurements at the level of the acceptance criterion for that constituent, while a method which is used to measure the quality or consistency of a material should provide accurate measurements throughout the range of possible outcomes. The requirements on a method that is utilized to release materials should be formulated on the basis of the risks of meeting specifications. Both the risk of false failure and the risk of false success should be considered.

The practical requirements of the method include considerations of the properties of the analyte to be measured, requirements for equipment, standards and reagents, and a vision toward constraints which may exist in commercial and regulatory laboratories. Added considerations include cost and efficiency. Likewise availability of equipment, standards, and reagents may influence the

choice of method. A method with high throughput may facilitate adequate replication and thereby, lower variability and risk.

12.1.4.2 Method Selection

Once the ATP is established, the method selection process begins with assessment of the alternatives. Relevant information from the ATP, the voice-of-the-customer (e.g., Quality Control laboratory or Regulatory Authority) is assessed so that desired operational intent of method for the end user is integrated into the selection process. Relevant testing platform/technology knowledge (use of methodology with similar product), control definition, noise elements, etc. are gathered to select the appropriate method(s).

A simple comparison of the traditional versus QbD process for method selection is outlined in Table 12.2. The key difference in the two approaches is that method quality and business performance is determined ahead of time in QbD, while it is typically derived from experience in a more traditional approach.

A framework is used to evaluate possible methods in terms of the categories above to select a suitable method for intended use from both the perspective of the CQA (quality benefit) and QC laboratory (business benefit), i.e., efficient in both cost and time.

Table 12.2 Comparison of traditional and QbD approaches to method selection

Characteristic	Traditional	QbD
Technology	Request to analyst driven, narrow option, often subject to personal preferences	Team-driven (analytical, production, quality, regulatory, end user). Use of entire analytical knowledge base, broad(er) option, aligned to customer needs
Accuracy, precision, linearity, range, QL, DL, specificity Method operating conditions to meet method characteristics	OFAT evaluations, experimental outcome determines acceptability	Acceptability predetermined by ATP requirements, DOE for robustness, LMM for intermediate precision
Operating space	Defined by development exercise	Defined by ATP then verified in development
Line-of-sight	Often limited to the current laboratory and up to the next planned study	Method life-cycle approach
Method comparison	OFAT, analyst driven, high potential of personal bias	Systematic, often involving scoring sheets, ATP driven
Selection criteria	Method quality and business performance focus	Total method quality focus

QL Quantitation Limit (LOQ), *DL* Detection Limit (LOD), *LMM* Linear Mixed Model, *OFAT* One Factor at a time

12.1.4.3 Method Risk Assessment

Risk assessment is a component of the quality risk management process defined in ICH Q9. The diagram (Fig. 12.2) illustrating the interplay of risk tools is given in the guidance (Guidance for Industry 2006):

The guidance (Guidance for Industry 2006) defines risk assessment as "A systematic process of organizing information to support a risk decision to be made within a risk management process. It consists of the identification of hazards and the analysis and evaluation of risks associated with exposure to those hazards."

Thus, risk assessment is the first stage of risk reduction, and is followed by risk control (via development) and risk review (via the control strategy). For analytical methods the hazards are unreliable results leading to poor decisions. Risk assessment is a process of identifying and controlling factors which impact measurements leading to poor decisions.

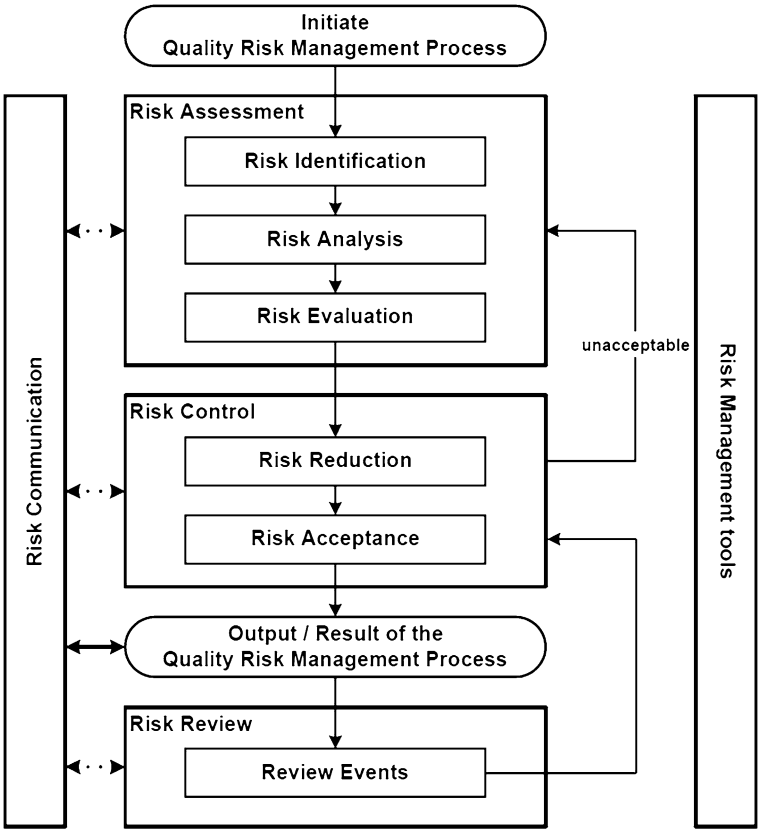


Fig. 12.2 Quality risk management process

Risk assessment begins with mapping the steps of the analytical "process." Every assay consists of several unit blocks whose sequence makes up the total assay process. For an ELISA, for example, these units include plate coating, sample capture, detection, substrate and data acquisition step, and wash steps. Additional subprocesses can be identified such as sample storage and preparation, analytical method preparation, and equipment supply and maintenance (see Fig. 12.6). The assay process should be considered the sum of the main process plus the subprocesses.

Each of these unit blocks has numerous input factors that affect the outcome. Some factors may have a greater impact than others; some may be more obvious than others. Systematic assessment of the input factors can be facilitated with tools such as an Ishikawa or fishbone diagram (see Fig. 12.7, Sect. 12.2.4).

Each step of the assay is represented by the intersection of the step description and the path to measurement reading. Factors, which may vary in each step, are identified and listed. These steps and their associated method factors are further evaluated based on prior knowledge to determine which may be subject to experimental verification.

Depending upon the complexity of the assay there are more or fewer factors which may impact assay quality and business performance. There are limitations on the ability to study all factors, and many of these factors are likely to have negligible impact on the assay. Consequently, tools such as cause and effects (C&E) matrices and failure modes and effects analyses (FMEA) are used to rate and rank factors.

It is important to note that risk assessment tools such as those illustrated are useful for ranking factors and guiding further exploration. These tools may also be useful to feed back into the method selection process. By their nature they are not definitive measures of factor severity and do not replace factual experimental results —based on prior knowledge or first principles, but not everything needs experimental verification. Of further note is that the risk assessment should consider less controlled (intermediate precision) factors such as technicians and reagent lots as well as controllable (robustness) factors such as temperature and time. Those controllable factors which have been earmarked as higher risk are further explored using univariate or multivariate DOE, while the less controlled factors may be assessed in the framework of measurement system analysis (MSA) using a design leading to variance component analysis or in blocked DOE designs.

Once the design space has been appropriately explored and established, remaining method risks can be evaluated in more detail using the FMEA tool. As each method block is analyzed one by one for potential failure modes and their impacts, the FMEA is particularly useful to assess risks in complex methods with various subprocesses. It is most powerful when applied to a limited number of risks that remain after development or after assay transfer since the risk assessment process is quite involved with rating each risk by severity, occurrence, and detectability in addition to exploring potential mitigation and control strategies.

Risk Factors are assessed in terms of severity, occurrence and detectability i.e., Risk Factor = Severity × Occurrence × Detectability (Sect. 12.1.4.3). A potency

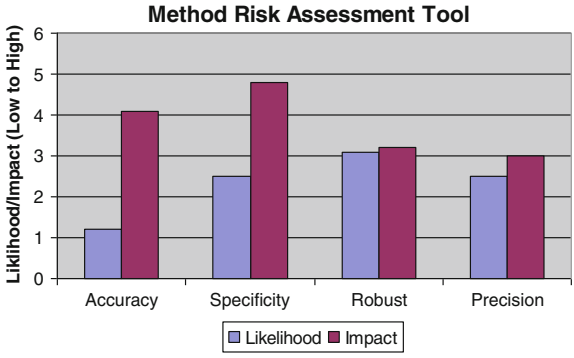


Fig. 12.3 Likelihood/severity impact assessment for purity assay selection. *Note* the components of total error (accuracy and precision) are included in the risk assessment

assay for a candidate vaccine is presented as an example in the case study that follows where the degraded antigen may negatively impact the potency assay output as well as product stability. Risk analysis is linked to the ATP used during the method definition process outlining that the method needs to be highly specific for the antigen with minimal reactivity to degraded antigen, and needs to assign potency in the range of 70–130 % of target while also being selective for native antigen over degraded antigen (stability program use). Total error must be 30 % or less with allowable precision of 15 %, this means that bias (accuracy) cannot be more than 5.3 %. Prior to method selection, the risk associated with the method's qualification/validation parameters, i.e., accuracy, precision, robustness, specificity are evaluated. Figure 12.3 graphically illustrates a risk assessment for this example. Each of the qualification/validation parameters are ranked in terms of likelihood and severity of impact on the outcome. From the ATP and the risk assessment, it is clear that keys to selecting an appropriate method are both accuracy and specificity, i.e., a minimally biased estimate of potency with discriminatory capability for degradants is critical for the method.

12.1.4.4 Design Space

The method design space like the process design space is "The multi-dimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality" (Guidance for Industry 2009). An approach to determining a design space is illustrated in ICH Q8(R2) and uses multifactor DOE together with acceptance criteria on quality attributes to define the region (overlapping contours) of acceptable quality. This approach is equally suitable to method development where ranges for assay method parameters are established which assure conformance of method quality attributes (CMAs and KMAs) to suitable quality and business acceptance criteria.

The successful application of DOE to the determination of the method design space is a multistage process. The first stage of the process is called screening, and involves the use of highly fractionated designs including Plackett–Burman designs to establish which method factors influence assay performance (Analytical Methods Committee 2013). Such designs have the capacity to examine many factors simultaneously. Factors which have been identified through the conservative application of risk assessment can be studied and potentially eliminated from more detailed experiments in a highly efficient manner.

Once some factors have been identified as important through screening, the design can be supplemented or a new design formulated to develop a mathematical model of assay response to variations in these influential factors. Mechanistic models can be developed when the mathematical relationship between method parameters (CMPs, KMPs) and response (CMAs, KMAs) is known, while useful estimates of an unknown model can be generated using designs which yield polynomial approximations. Designs such as the Box–Behnken or central composite designs have the power to formulate second and third order polynomial models which have superior predictive properties since they consider two and three factor interactions among other aspects (Ferreira et al. 2007).

Another key to successful performance modeling is the identification of responses which best relate to method quality. When the objective in the development of an HPLC method is to discriminate product and impurity peaks, standard outcomes (CMAs, KMAs) such as resolution may fail to identify conditions which simultaneously separate chemically dissimilar components. This is because resolution is insensitive to the impact of asymmetric peaks. A more direct measure of resolution may be the minimum distance between the back and front of adjacent peaks. Another example is in binding assays when the variability of the final result (sometimes potency) is indirectly related to the slope of the concentration response curve. Use of indirect measures of quality characteristics such as precision may result in more efficient experimental design strategies.

Once a mathematical model of a method quality attribute has been obtained, it can be utilized to identify the region of the method parameters which is predicted to yield acceptable method performance. As depicted in ICH Q8 (R2) (Guidance for Industry 2009), this region gives estimated responses which satisfy performance acceptance criteria (e.g., %CV < 20 %). For simple visual communication, regions of fixed responses can be determined from the mathematical model and displayed as a contour plot (Fig. 12.4).

Thus, the unshaded region in the illustrative plot might correspond to the limits in which the predicted variability of the method is <20 % CV. Ranges on the method parameters can be set by inscribing a rectangle which effectively covers the unshaded space, or by operating the assay within the mathematically derived region. However, this method of contour regions, although readily accessible through standard DOE software, fails to satisfy the definition of design space. In ICH Q8 the design space is specified to "assure" quality (Guidance for Industry 2009). The probability of meeting a performance parameter acceptance criterion at the edges of the contour region is ≤50 %. This is because the contour boundary

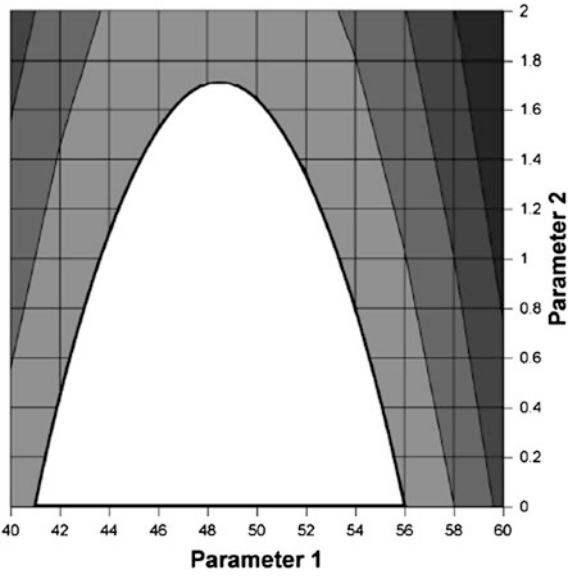


Fig. 12.4 Example contour plot

represents the estimated average, without regard to uncertainty. Modeling and simulation have helped to define regions of the method parameters which assure a high probability of satisfactory performance of the method.

12.1.4.5 Control Strategy

The method control strategy contains two key elements: (1) control of critical method parameters (i.e., those found to adversely impact test results); and (2) those that are determined to be useful as monitoring tools during routine method use. The output of risk assessment is used to determine 1 and the output of robustness DoEs can be used to determine 2.

Additional method control comes from long-term monitoring of method quality and business performance. System suitability tests are monitored long term and the tests limits are used as control criteria for drift within acceptable ranges. ICH guidance Q2(R2) states "one consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution tests) is established to ensure that the validity of the analytical procedure is maintained whenever used" (ICH 2000). The robustness DoE therefore can be used to define system suitability test (SST) limits (Guidance for Industry 2000) if additional data from the method robustness DoE runs is assessed e.g., slopes, peak resolution etc. The parameter responses from the worst-case results of the DoE are used to determine SST limits.

NOTE SST limits should only be determined once a method is considered robust. This ensures that if a result is obtained when a SST result is at or near its control limit, *there will be confidence that the analytical result is valid.*

The ATP forms the boundaries that help define the control strategy, i.e., the desired operating space that is monitored for controlling the method's quality and business performance during routine use. Once method optimization is completed, formal limits based on assessments of method robustness (DOE) and intermediate precision (Linear Mixed Model with random effects (LMM)), can be used to set up the method control limits and defined operating ranges. Method parameters that are not sufficiently robust must be sufficiently controlled for the method to operate within the acceptable quality performance space, i.e., controlled operating limits.

A second, yet equally important part of the control strategy is the real-time monitoring of the analytical method once it is validated and implemented in the QC laboratory. Analytical Method Maintenance (AMM) should be a formalized process and managed as part of the Validation Master Plan (VMP). Section 12.1.4.6 provides details for AMM.

A Linear Mixed Model with random effects (REML) study of noise factors (external factors contributing to overall method variability e.g., reagents, analysts, instruments) during method development can be used for understanding variance components due to these noise factors. Understanding these factors is important in terms of continuous improvement for the method. For example, if the method is validated with an intermediate precision assessment (e.g., 5 %) that is acceptable for the ATP, analysis of the individual contributing noise components can give insight into future improvements. For example, the LMM analysis reveals 25 % of total precision variability is due to instrument detectors (possibly due to calibration variation), more precise detectors can be used or a more controlled detector calibration program initiated to improve quality or performance.

Additional control is developed through system suitability requirements, some of which can control for noise factor influence prior to testing. From the example above, if detector calibration was found to be the root cause of the large detector variation component of precision, development of a system suitability check for detector response could be built into the method system suitability requirements.

Comparability exercises should be based on equivalency assessments and not lack of evidence of significant difference. The use of a two one-sided test (TOST) approach for equivalency testing is ideally suited to the ATP concept. The ATP precision and accuracy requirements are used to evaluate the suitability of the predetermined acceptable difference in results based on prior knowledge that will be used to assess the TOST data when performing equivalency evaluations during method replacement or method transfer activities. The term (θ) is used to describe the acceptance criterion, specifically the limit outside which the difference in mean values is considered to be both practically and statistically different (Vander Heyden et al. 2001).

Implementation of the control strategy during method development and optimization leads to identifying suitable method parameters used for system suitability i.e., daily method control. The control strategy also includes long-term control that is achieved through AMM (see Sect. 12.1.4.6).

Two categories of tools are used to develop/implement the control strategy and AMM. The first set consists of risk assessment and statistical tools used for method

optimization and understanding, i.e., DoE's for robustness and precision understanding; FMEA's, C&E matrices, and fishbone diagrams for critical factor determination and understanding. The second set of tools, statistical in nature, is used for daily method monitoring to assure the maintenance of the method in the desired state, i.e., control charts of standards, controls, SST values, etc. In addition to routine control chart monitoring, periodic reviews of performance against validation status and change control reviews are part of the control strategy for AMM.

Once a method has been developed and the method operating procedure has been written, the development laboratory typically undertakes a series of studies to establish that the method is suitable for its intended purpose. Although the terminology may differ from organization to organization, these studies are usually called method qualification and method validation. Method qualification is executed for the purpose of demonstrating that an analytical method is scientifically sound, suitable for its intended purpose, and capable of generating reliable results (Guidance for Industry 2011; Vander Heyden et al. 2001; Limentani et al. 2005; Ritter et al. 2004) Qualification is a less rigorous process than validation, but similar in that it is used to ***assess*** a method at the end of or concurrent with development to determine if the method's design and quality performance are suitable for its intended use. In contrast, method validation *confirms* a method's expected performance with predefined assessment criteria that include every data element required as per Q2(R1) (ICH 2000). Method validation meets all Q2R1 requirements. Method qualification is based on Q2R1, but may not include all data elements such as intermediate precision.

Since methods are used throughout product development to guide decisions, method qualification may be considered a dynamic process. Product development decisions are impacted by the strength of the design of the development experiment and the reliability of the measurement tools. Thus, a method should always be "qualified" to satisfy its intended purpose of supporting product development.

One of the tacit principles of QbD is that quality should be built into the process rather than empirically derived from performance of the method. The formal validation is an empirically derived confirmation of a method's performance. The method validation may be otherwise used to inform the laboratory about the variability characteristics of the method under conditions of long-term use. This is illustrated in *USP <1033> Biological Assay Validation* (Guidance for Industry 2008). The results of a properly designed validation may be used to build quality into the assay (in this case a bioassay) through appropriate formatting. The example taken from the chapter is shown in Table 12.3.

Table 12.3 Impact on formatting for bioassay variation

Reps (n)	Number of runs (k) (Geometric coefficient of variation %)			
	1 (%)	2 (%)	3 (%)	6 (%)
1	7.2	5.1	4.1	2.9
2	6.4	4.5	3.6	2.6
3	6.0	4.2	3.4	2.4
6	5.7	4.0	3.3	2.3

The percent geometric coefficient of variation is shown for different combinations of numbers of runs (k) and numbers of replicates within a run (n). If the precision cannot exceed 5 % to be fit for purpose, the laboratory can either perform the bioassay in three runs with a single replicate in each run, or in two runs each with two replicates. This represents the "design space" in the replication factors of the bioassay. The format may be different for different uses of the assay. For example, more precision (and thereby less uncertainty) may be preferred for the calibration of a working standard.

The combined assay development (robustness), qualification and validation represent the validation package. Qualification and validation runs can be combined to obtain a more powerful knowledge assessment of the quality performance characteristics of the assay.

12.1.4.6 Analytical Method Maintenance

To facilitate continuous improvement of analytical methods, monitoring the controlled state is achieved through an AMM program (Guidance for Industry 2008). AMM programs provide a real-time view of the analytical method's quality (and perhaps business) performance based on monitoring and assessing the method's state of control. Thus, they can provide assurance that the method is being maintained as suitable for intended use post validation. The cornerstone of AMM is the test method Control Chart, along with how reference standards and other critical reagents are monitored.

- Control charting

A reference standard is an in-house standard for the material being tested. For example, a control lot of polysaccharide X (reference standard) is run in each hexose content assay along with the newly manufactured lot of polysaccharide X. Monitoring the reference standard values gives an indication of the method's quality over time (short- or long-time frame). This type of control chart will monitor the method's real intermediate precision over time as the components of the method's precision consist of the typical components that contribute to the method's variability (e.g., operators, days, instruments, reagents). Additional monitoring of method quality characteristics, such as slopes, baseline noise values, etc., is also useful.

If the method control chart is combined with the manufacturing control chart for the commodity tested, a complete set of manufacturing process control data is available. A combination control chart (overlay of test method chart on manufacturing control chart) allows for rapid identification of laboratory or manufacturing outliers and for rapid action to maintain overall process control. The AMM control chart thus forms a direct linkage between QbD for analytical methods and QbD for manufacturing processes.

- What to chart to be efficient but have enough information to troubleshoot when necessary

The ideal material to use in control charting an analytical method is a reference material from the same process producing the test article. If this is not available, then a positive control sample that is run through the method as a sample can be used. This reference material has an assigned value and expiry; the quality performance of this reference material then is monitored in every run for its ability to stay within the ATP predefined accuracy and precision requirements. Deviations from alert limits or trends in direction of values within the accuracy and precision limits are identified and acted upon in a timely manner leading to real-time control of method quality. The monitoring of this control has added advantage as a troubleshooting tool in OOS investigations as well as supporting process monitoring.

- How to incorporate continuous improvement for methods in validated QC space?

A second key component of AMM is monitoring and control over changes to the method or its components over time. The ATP contains specific quality and business requirements that govern acceptability of method changes, i.e., operational limits within which a change must operate. An allowable difference for any method component change can be determined based on the ATP and documented quality performance of the method from validation and routine use (active monitoring of control chart). Then use of accuracy and precision requirements (ATP defined) can be used to design equivalency studies that can be analyzed using a two one-sided test (TOST) approach.

- Incorporation of Continuous Improvement, Method Changes through Life-cycle

The control strategy should take into account the analytical method life-cycle. Over time and routine use of the method, changes inevitably occur that need to be assessed for impact to the validated state of the method i.e., its ability to stay within the ATP. Method components may need to be changed or replaced e.g., columns, detectors, reference materials, etc. Other changes may include transfer to other laboratories or even complete replacement of the method using an alternative technology.

The TOST approach for equivalency testing (Vander Heyden et al. 2001), with criteria based on ATP and allowable difference (θ) between licensed and proposed new method, can also be used if a method is to be transferred or replaced at some point during its life-cycle. This approach allows for difference in the methods being compared based on prior knowledge and intended use with a limit based on both statistical as well as practical differences that is used to determine equivalence.

- Reference standard and critical reagents

A reference standard and critical reagents program should be established to assure continuity of scale and maintenance of the method range. The units of the reference standard define the scale of the method. This may be in units of

concentration such as a protein standard, or in units of a primary reference standard as in an in vitro or in vivo potency assay. The reference standard may be obtained externally from a compendial or commercial source, or manufactured internally.

If the reference standard is obtained externally it is qualified for use in the assay. The qualification should be designed to demonstrate that the reference standard is fit for use in the intended method. This may be carried out as a comparability exercise using a TOST approach for equivalency testing. An internal reference standard program should ideally combine the use of a primary reference standard with replaceable working standards. In this case, a working standard is qualified against the primary standard. This is in contrast to a program where each new reference standard is qualified against its immediate predecessor (serial qualification). Unlike a primary reference standard program, serial qualification is subject to method drift in scale as well as propagation of measurement uncertainty.

The qualification of a new reference standard can be carried out in one of two ways: (1) demonstration of equivalence of the new standard to its comparator (the primary or previous reference standard); or (2) calibration of the new reference standard to its comparator. The choice of approach depends upon the variability in manufacture of new reference standards. When the manufacture of new standards is variable, demonstration of equivalence of a new standard is difficult and may result in an apparent shift in manufacturing history and poor manufacturing process capability. Calibration of a new reference standard should be carried out in such a way as to minimize the uncertainty of the assigned value for the standard. If coupled with a primary standard the calibration also serves to monitor the stability of the primary standard. If serial calibration is performed, the new standard can be assayed as a test sample in a sufficient number of runs to minimize uncertainty.

Stability of a reference standard should be assessed to minimize the impact of drift. As highlighted previously the primary standard stability can be assessed as part of the qualification of new working standards. Stability of a working standard can be routinely monitored through some absolute characteristic of the standard. Trending of method system suitability parameters reveals changes in the standard. For a potency assay the 50th percentile of the reference curve or response at one or more dilutions may be used to monitor stability. Drift in the method control is not a sensitive measure of standard stability as the control may be unstable. Stability assurance should be considered in conjunction with stability control. Thus, the standard might be a lyophilized preparation of the manufactured material and/or may be held under colder conditions.

Qualification of critical reagents provides assurance of continuity of the method range. As with the reference standard a critical reagent may be tested for equivalence to a current reagent or titrated to a level which ensures equivalence. Since the range of the method is defined by the units of the reference standard, qualification of a critical reagent should be performed on this material.

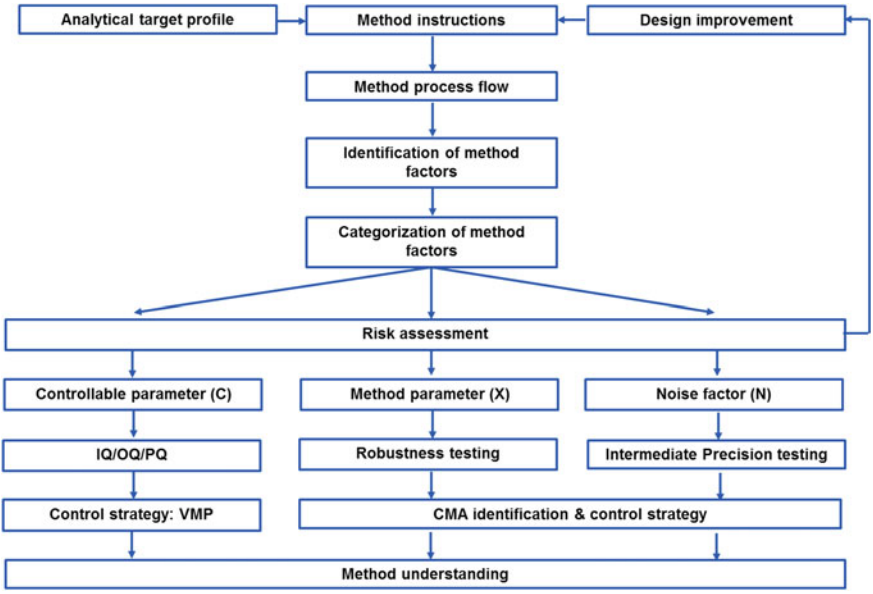


Fig. 12.5 Conceptualized method development process

12.2 Case Study

12.2.1 Introduction and Background Driving QbD Development of a Potency Assay

The case study presented below describes in detail the application of QbD principles for the selection and development of a Potency Assay for a hypothetical protein-based vaccine. Figure 12.5 presents graphically a method development process. Our case study will examine in depth several of the steps in the diagram to demonstrate the application of QbD principles to analytical method development.

12.2.2 Analytical Target Profile

Prior to method selection and development, details of what the method needs to do (has been requested to do), how it should perform, and how it will be controlled are documented and an ATP formalized. A team approach is implemented to develop the ATP with representatives from the various groups that will be directly impacted by use of the method, i.e., development, analytical, regulatory, and quality. Table 12.4 summarizes the background information that needs to be collected and utilized by the team that will guide the ATP definition.

Table 12.4 Background information to needed to facilitate development of ATP for potency method

Prior knowledge	Efficacy of candidate directly related to protein content
	Similar licensed DS/DP manufactured on site has potency assay based on same technology
Purpose	Potency assay used for measuring antigenically active protein components with respect to the total protein amount
Method intent	State-of-the-art immunochemical quantification of antigen for lot-release and stability potency testing of drug substance and drug product
Method selection	Double antibody sandwich ELISA
Reportable value	Antigenicity (%) = Antigen content/protein content
Method quality performance criteria	ICH Q2(R1) for potency assay
Method business performance criteria	Throughput, ease of use, investigation resources
Stability indication	Assay should be capable of discriminating native antigen from degraded antigen

Table 12.5 summarizes the ATP for the analytical method of the case study. The method is an ELISA method to measure potency of the bulk drug substance (DS) that is part of a multicomponent drug product (DP) vaccine.

One key element of QbD application to analytical method development is the systematic identification and use of prior knowledge (also shared with classical approach). Selection requires complete understanding of the technical aspects as well as understanding of quality and business capabilities of the method in order to align the method with the ATP. Prior knowledge for our Potency Assay is summarized below (a asterisk indicates items assessed through experimental design to highlight application of QbD principles):

1. Prior Knowledge of the sandwich ELISA: theoretical considerations
   The Sandwich ELISA measures the amount of antigen between two layers of antibodies (i.e., capture and detection antibody). The antigen to be measured must contain at least two antigenic sites capable of binding to antibody, since at least two antibodies act in the sandwich.
   Either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in Sandwich ELISA systems. Monoclonal antibodies recognize a single epitope that allows fine detection and quantification of small differences in antigen. A polyclonal is often used as the capture antibody to pull down as much of the antigen as possible.
   The advantage of Sandwich ELISA is that the sample does not have to be purified before analysis, and the assay can be very sensitive up to 2–5 times more sensitive than direct or indirect and very accurate and reproducible.

Table 12.5 ATP potency assay (validation requirements per (USP 2014))

DS mfg requirements and targets	Method characteristic	ATP criteria
Purity: NLT 95 % Potency: 100 ng mL^{-1} Range: 70–130 ng mL^{-1} Target 100 ng mL^{-1} Sampling: minimal sample volume due to high mfg cost Stability indicating	Accuracy	Bias NMT 5 %
	Precision	Intermediate (Ruggedness): %RSD NMT 20 %
	Total error (% Bias + 1.65 × % RSD)	±30 %
	Specificity	Specific to native antigen
		Reduced reactivity with degraded antigen
		NMT 5 % bias due to excipients and process residuals
	Linearity	$R^2 > 0.98$, normal distribution of residuals
	Range	70–130 % of specification
	Robustness	NMT 20 % contribution of any method factor to overall test method variability
		All other factors controlled through specific method settings
	System suitability	GMT reference sera NLT 83 % (antigenicity)
		Plate background NMT 0.20 Abs units
		B parameter NMT 1.21 (from upper 90 % CI)
		D parameter NMT 2.81 (from upper 90 % CI)

2. Conceptual design of the sandwich ELISA: a good ELISA assay shows…

- Specificity toward the analyte to be quantified*
- S-shaped dose-response curve (OD versus dilution level or concentration)*
- Clearly defined upper and lower asymptotes with ≥3 inter-dilution points in the linear working range of the standard curve*
- Acceptable parallelism between standard and samples
- Acceptable precision and accuracy balanced to meet criteria for Total Error and established specifications*
- Stability-indicating properties*
- Optimized conditions for sample preparation, dilution scheme(s) (incl. sample pre-dilution), concentrations of Ab, Ag, blocking reagents…*
- Identified major sources of variability with respect to sample preparation, incubation durations and temperatures, stability of coloration, edge effect…*

3. Conceptual design of the sandwich ELISA: Development success criteria

   - Efficient antigen binding and assay specificity towards antigen
   - Decreased binding with denaturated/partially degraded antigen (stability indication)
   - Background <0.2 AU*
   - OD working range >2.0 AU*
   - No hook effect above antibody saturation*
   - Assay sensitivity and linearity*
   - Assay easy to operate in routine

4. Critical reagents:

   - Selection of the reference material used for sample quantitation:
   - Long-term stability ⇒ use of lyophilized material when possible
   - Calibrated unitage in protein content ⇒ use method with known error (bias/RSD)
   - ELISA response characterized ⇒ use control chart (e.g. on mid-point)
   - Control strategy of reference material: check for cake appearance? Resuspension time?
   - Selection of the internal control used for system suitability:
   - Long-term stability ⇒ use of lyophilized material
   - Time difference between manufacturing of the reference and control
   - ELISA response characterized ⇒ use control chart (e.g., on antigen content)

12.2.3 Method Selection

Two candidate methodologies were available to the laboratory as potential potency assay formats. A simple ranking analysis tool was used as an aid in the choice of two alternative methods. The tool has three steps followed in sequence to evaluate (1) CMAs, (2) KMAs, and (3) prior knowledge of the two candidate methods against the ATP.

The evaluation can be stopped at any step at which a quality difference is detected. In the example below (Table 12.6), such a quality difference was already detected after step 1, the CMA evaluation. During the evaluation process, a priority value (Table 12.6: "Priority") and a score value (Table 12.6: "Score-2") were assigned to each method characteristic for each of the methods to arrive at an overall rating (Table 12.6: "Rating") for each. The total rating at the end of step 1 (Table 12.6: "Total") pointed to the sandwich ELISA as a better fit for meeting all the quality attributes desired from the ATP, i.e., the total rating score for ELISA based solely on CMAs was 140 points higher than that of the cell based assay. In cases where the differentiation at the CMA level may not be as apparent, additional assessment of KMA scores, and/or prior knowledge can be used as part of the three-step process.

Table 12.6 Method selection factor ranking analysis

Characteristic	Method	Priority	Score-1	Score-2	Rating
Accuracy	ELISA	1	100	100	100
Bias NMT 5 %	Cell assay			100	100
Specificity/discriminate degradant	ELISA	1	100	100	100
	Cell assay			50	50
Precision	ELISA	0.5	100	100	50
	Cell assay			50	25
Linearity	ELISA	0.1	100	100	10
	Cell assay			50	5
Range	ELISA	0.5	100	100	50
	Cell assay			100	50
Robustness	ELISA	1	100	100	100
	Cell assay			50	50
System suitability	ELISA	1	0	100	100
	Cell assay			100	100
Total	ELISA	**520**			
	Cell assay	**380**			

Priority is based on method: *Low* 0.1, *Medium* 0.5, *High* 1.0
Score 1 Indicates if method meeting CMA from ATP is independent (*No* 0, *Yes* 100)
Score 2 Ability to meet ATP requirement: *not met* 0, *partially met* 30, or 50, *fully met* 100
Rating = Score 2 × Priority

Prior company experience with a similar product and analytical expertise with the sandwich ELISA format were important factors in the method selection process. Due to successful development and implementation of a sandwich ELISA as the potency assay for similar product, the selection process was abbreviated to documenting previous experience on the same format.

However, customer requirements and expectations still were systematically assessed as shown in Table 12.7. The impacts of customer (business) requirements such as specificity, transferability, or critical reagent availability were evaluated with respect to their importance for quality parameters such as accuracy, and precision. The impact of quality parameters on method outputs that are seen by the customer was divided into scores of low (+), medium (++), and high (+++). Associated number scores were then used to calculate a "critical to customer (CTC)" score to rank-order the quality parameters. Subsequent risk assessment then focused on the highest ranked quality parameters, i.e., those with the highest CTC index.

The Quality Function Deployment matrix is a decision-making systematic process used to incorporate customer requirements into every aspect of the design with respect to critical functionalities. In analytical QbD, the matrix can be designed so that measurable functional requirements selected according to the ICH Q2 guideline (ICH 2000) on method validation are rated on a scale of 1 (weak), 3 (moderate) or 9 (strong). Rating the relationship between customer and functional requirements is

Table 12.7 Quality function deployment matrix for antigenicity ELISA

	Quality Function Deployment matrix for antigenicity ELISA						
Functional requirements / Customer's requirement	Specificity	Accuracy	Precision	Linearity and range	LOD/ LOQ	Robustness	Stab-indication
Specific of the antigen to quantify	9	3	1	9	1	1	9
Quantitative	9	9	3	9	3	1	9
Simple to use and high throughput	1	1	3	1	1	9	1
Compatible with complex matrices	9	9	1	3	1	1	1
Predictive of antigen 3rd structure	1	1	3	1	1	1	9
Transferable	1	3	3	1	1	3	1
Critical reagent available	3	1	1	3	1	3	1
Cheap and robust	1	1	1	1	1	9	1
Critical to Customer (CTC)	2,187	729	81	81	3	729	729
Method performance criteria	Specific, used for IPC/DS/DP	Total error within ±30 %	<20 % (IP)	Range for quantitation	Not critical	Risk-assessed robust assay	Used for shelf-life

then used for prioritization of development activities using a Pareto chart and helps focusing resources on design aspects that have the greatest impact on method performance and customer satisfaction.

12.2.4 Risk Assessment

Once the basic method was selected and CTC scores of quality parameters were established, the first step of risk assessment focused on mapping out the overall assay process and the subprocesses (Fig. 12.6). Several blocks within the main stream of carrying out the assay were identified starting with Raw Material Storage and ending with Data Acquisition. Additional subprocesses that feed into the main assay process were identified as well, for example, the sample preparation and equipment operation.

As a detailed risk assessment of the entire system was too great of a task to be carried out within the framework and timelines of practical assay development, risks of subprocesses with minimal direct interaction with the main assay were assessed in less detail. Systematic risk assessment could then focus on the assay itself. The sample/reference dilution which was mapped to the sample preparation subprocess was still included in the risk assessment since it was closely associated with carrying out the assay. An Ishikawa Diagram (aka Cause and Effects or Fishbone Diagram) was used to brain-storm inputs of the assay process that could affect measured results (Fig. 12.7). Input factors were assigned into six categories:

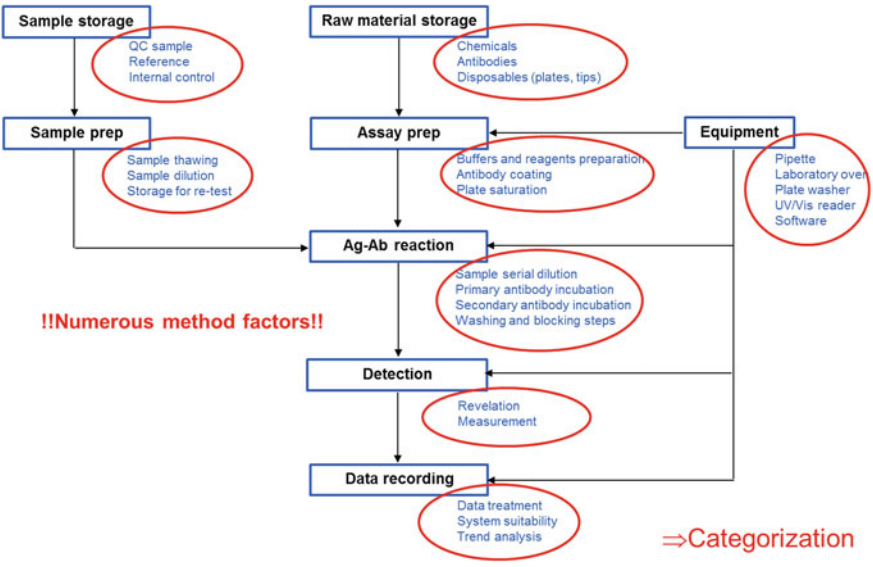


Fig. 12.6 Process map displaying the immunoassay steps and associated input sub-processes

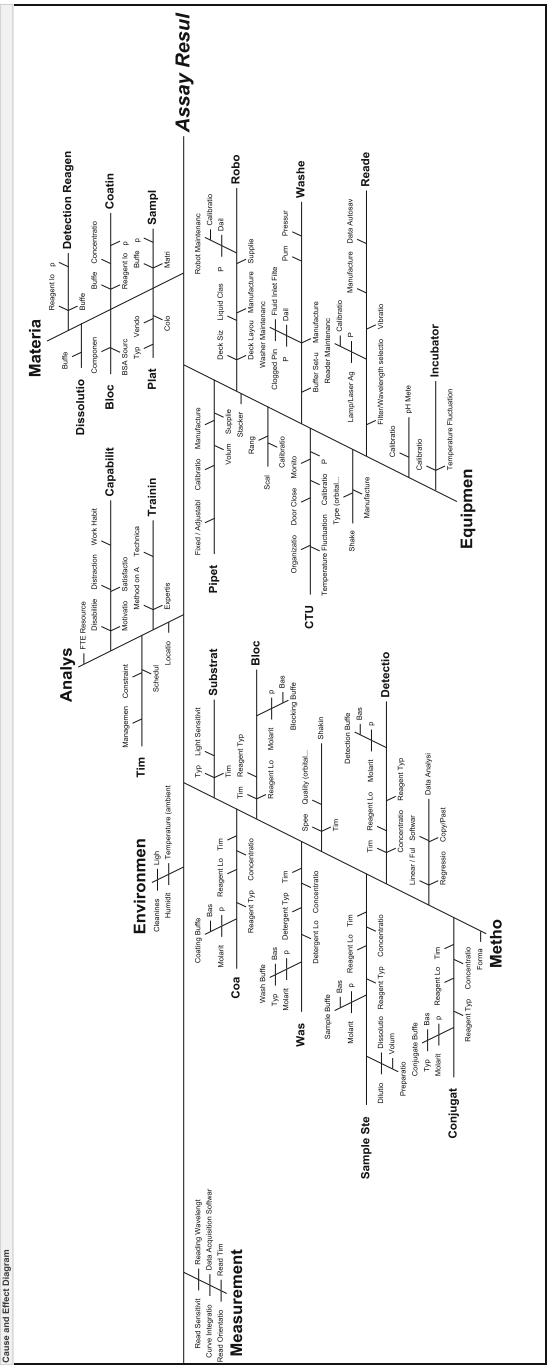


Fig. 12.7 Ishikawa diagram (aka Fishbone or Cause and Effect diagram). Method steps are assigned to six categories. Individual input factors are assigned to the respected method steps and categories

Table 12.8 Example of a cause and effects (C&E) diagram to determine impacts of assays risks on ATP requirements

Method parameter = X	Specificity	Linearity	Sensitivity	Precision	Accuracy	Impact	Rank
Sample/reference dilution		5			5	25	H
Antibody dilution			5		3	15	M
Use of automated versus manual wash				5		5	L
Ab coating duration				3	3	9	L
Ag–Ab reaction duration		5		5	3	75	H
Ag–Ab reaction temperature	3			5		15	M
Blocking reagent type	3				3	9	L
Blocking reagent concentration	3				5	15	M
Revelation time		3		3		9	L
Coloration stability		3		3	3	27	H
Reading wavelength			1	3	5	15	M

Each risk is scored by low, medium, and high impact on each requirement. The final risk score is the product of all impact scores. Final risk scores are categorized as low (1–9), medium (10–20), and high (>20)

Analyst, Equipment, Method, Measurement, Environment, and Materials/Supplies. Individual blocks in the process map such as "Coat, Detection…" became secondary side chains ("spines") within each of the six main categories. Lastly, individual factors were visualized by tertiary side chains within each block, for example "reagent concentration, incubation time…".

With the process map and the Ishikawa diagram as a foundation, risks associated with individual inputs/steps were listed and ranked using a Cause and Effects Matrix (Table 12.8). Initially, the potential impacts of factors onto each CMA (identified in the ATP, see Sect. 12.2.2) were translated into individual risk scores and summed up into final risk scores. Final scores were triaged into high, medium, and low risks based on a cut-off matrix. High level risks (sum > 20) and their interactions were then studied further in multifactorial designed experiments (see Sect. 12.2.5) to explore and optimize the design space.

Risk factors ranked as "high" in the C&E diagram (matrix) were explored further by FMEA in order to select appropriate factors to study in development (see Table 12.9 and Fig. 12.8).

Highest risks were associated with inappropriate choice of assay conditions for sample dilution schemes, antigen-antibody reaction time, substrate incubation time, and antibody dilution factor. Incoming quality control of substrate lots were also

Table 12.9 Failure Modes Effect Analysis (FMEA): High risks as determined in the C&E matrix were analyzed for potential failure modes and causes

	Process step	Key process input	Potential failure mode	Potential failure effects	Potential causes	SEV	OCC	DET	RPN	Actions recommended
C&E risk rank	What is the process step?	What are the Key Process Inputs? (KPIV's)	In what ways can key Inputs go wrong? (process fail to meet requirements)	What is the impact on the key output variables (customer requirements) or internal requirements?	What causes the key Input to go wrong? (How could the failure mode occur?)	How Severe is effect to the customer?	How frequent is cause likely to Occur?	How probable is detection of cause?	Risk priority # to rank order concerns	What are the actions for reducing the occurrence of the cause, or improving detection? Should have actions on high RPN's or Severity of 9 or 10
H	Sample Prep	Sample/reference dilution	Inaccurate	Inaccurate result	Calculation error	9	3	1	27	Predetermined dilution schemes
					Pipet Inaccuracy	1	1	3	3	Regular pipet calibration
					Tube mix-up	9	1	3	27	Color codes, visual place maps
					Chosen conditions	9	3	9	243	Study
H		Ag–Ab reaction duration	Too short/long	Reduced robustness	Analyst error	3	1	1	3	Timer and recording templates
					Chosen conditions	9	3	9	243	Study
H	Substrate incubation	Coloration stability	Too long incubation	Reduced robustness, assay failure (no data for customer)	Analyst error	3	1	1	3	Timer and recording templates
				Reduced robustness, assay failure (no data for customer)	Chosen conditions	9	3	9	243	Study
			Light sensitivity	Reduced robustness, assay failure (no data for customer)	Exposure to sun light	3	1	1	3	Incubate in the dark (AP requirement)
			Lot quality	Reduced robustness, assay failure (no data for customer)	Manufacturer issues	9	1	9	81	Incoming QA test

(continued)

Table 12.9 (continued)

	Process step	Key process input	Potential failure mode	Potential failure effects	Potential causes	SEV	OCC	DET	RPN	Actions recommended
C&E risk rank	What is the process step?	What are the Key Process Inputs? (KPIV's)	In what ways can key Inputs go wrong? (process fail to meet requirements)	What is the impact on the key output variables (customer requirements) or internal requirements?	What causes the key Input to go wrong? (How could the failure mode occur?)	How Severe is effect to the customer?	How frequent is cause likely to Occur?	How probable is detection of cause?	Risk priority # to rank order concerns	What are the actions for reducing the occurrence of the cause, or improving detection? Should have actions on high RPN's or Severity of 9 or 10
M	Antibody Prep	Antibody dilution	Inaccurate	Reduced Robustness	Calculation error	3	3	1	9	Predetermined dilution schemes
					Pipet Inaccuracy	1	1	3	3	Regular pipet calibration
					Chosen conditions	9	1	9	81	Study
M	Capture and detection	Ag–Ab reaction temperature	Too high/too low	Reduced Robustness	HVAC or incubator failure or capability	3	1	1	3	Use and qualification of incubators
M	Block	Blocking reagent concentration	Too low/high	Reduced Robustness	Calculation error	3	1	1	3	Check boxes in protocol (required versus added)
M	Plate Read	Reading wavelength	Off target	Reduced robustness, Assay failure (no data for customer)	Analyst error	9	1	1	9	Check boxes in protocol (required versus added)
				Reduced robustness	Instrument capability	3	3	1	9	Specify instrument, explore design space
				Reduced robustness	Instrument maintenance	3	3	3	27	Maintenance schdule, performance checks
				Reduced robustness	Chosen conditions	9	1	1	9	Follow manufacturer's recommendation

These were ranked on a scale of 1 (low risk), 3, (medium), and 9 (high) by severity of impacts, occurrence/frequency of failures, and detectability (*1* easy to detect, *9* difficult to detect). The product of individual rankings resulted in a risk priority score (RPN)

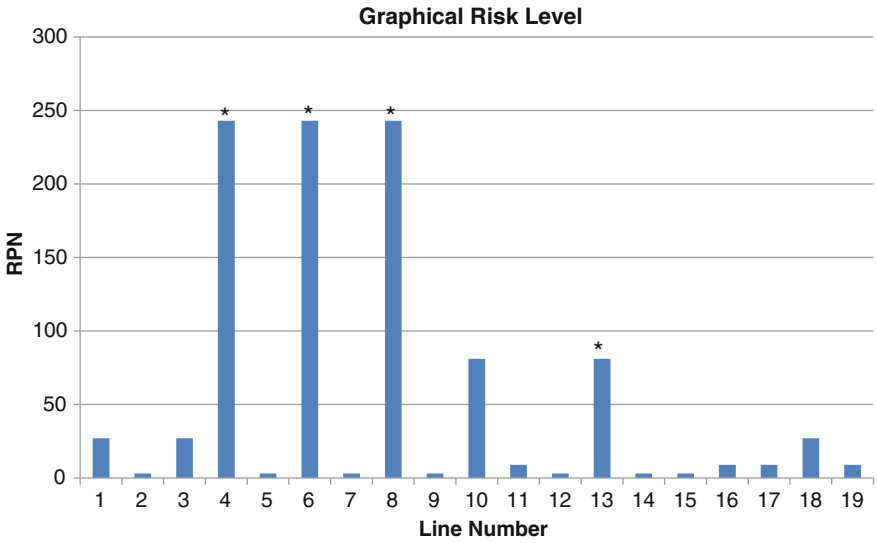


Fig. 12.8 Risk Priority Numbers (RPN) were calculated as the product of severity, occurrence, and detectability rankings and graphed against the risk ID. Highest risks as indicated by "*" were studied in DOEs

found to be important but could not be controlled via the design space. Based on their risk, these factors were studied during development.

12.2.5 Design Space

The first stage in ELISA development encompasses the selection of antibodies. Drivers in selection include assay-related technical rationale, such as ability of antibodies to show antigen degradation and to ensure appropriate assay quality and business performance, as well as external assay factors, such as in vitro/in vivo neutralizing properties. Selection of antibodies relies on the design of the assay, e. g., Sandwich versus competitive ELISA or direct versus indirect detection formats. It is widely acknowledged that epitope-directed monoclonal antibodies exhibit higher specificity and better discriminant properties toward change in antigen conformations.

In the case study below, an indirect Sandwich ELISA format was selected with antigen capture onto polyclonal antibody-coated plates and monoclonal antibody coupled to HRP-conjugated secondary antibody for antigen detection and quantitation. Peptide epitope candidates were identified from the antigen primary structure using in silico prediction algorithm and corresponding peptides were synthesized and used to immunized rabbits. Six different monoclonal antibodies were raised then screened for their ability to distinguish between native and degraded antigen.

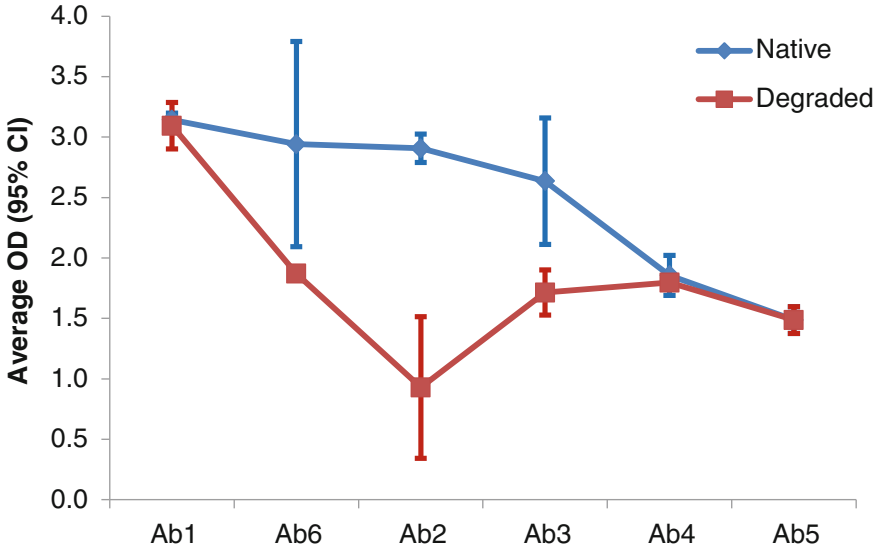


Fig. 12.9 Antigen response plot obtained with different monoclonal antibodies directed against six epitopes

The plot in Fig. 12.9 shows the monoclonal antibodies ordered by the response to the native antigen. ANOVA shows a significant interaction between species (native and degraded) and antibody (p-value < 0.0001). Pairwise comparison between species shows that the monoclonal antibody directed against epitope 2 "Ab2" exhibits appropriate stability-indicating profile. Overall the Ab2 ELISA shows high specificity, selective reactivity to native antigen and low background. Interestingly

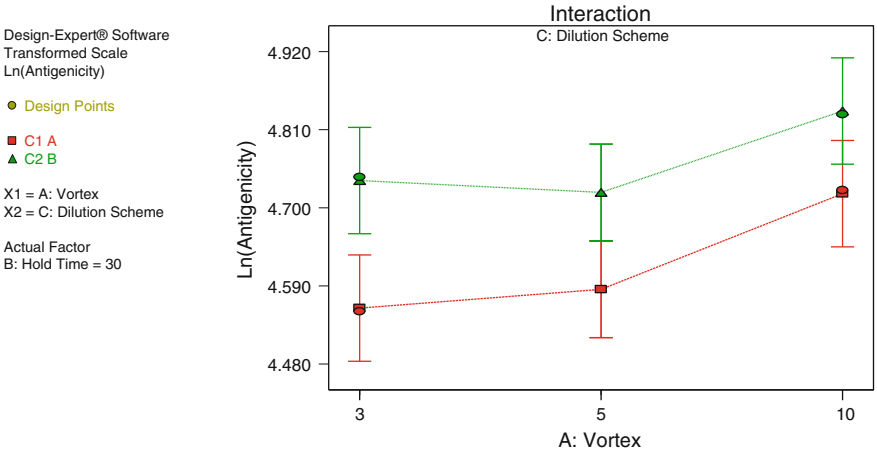


Fig. 12.10 Influence of vortex duration and dilution scheme on sample antigenicity

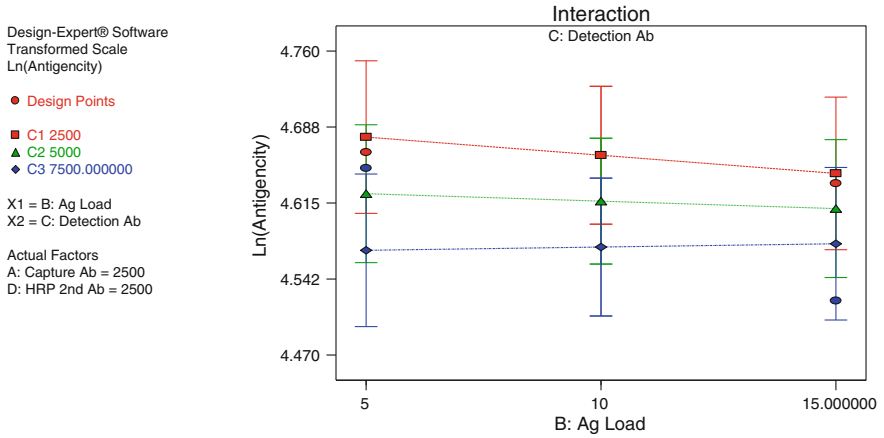


Fig. 12.11 Influence of detection Ab2 concentration on sample antigenicity

"Ab2" exhibits neutralizing properties towards the antigen in vitro as measured by antigen-antibody neutralization assay on blood specimen.

Assay robustness was evaluated according to the science- and risk-based approach described in the above sections. The assay process map was brainstormed and potential Critical Method Factors (CMFs) were identified using the Cause and Effect diagram and risk ranked by Failure Mode and Effect Analysis. Design of Experiments was used to assess the influence of potential CMFs on assay robustness. Potential CMFs were clustered in independent DOEs according to their ability to impact sample integrity or assay quality/business performance. Response factors were selected accordingly, i.e., the antigenicity of the sample and/or midpoint of the standard quantitation curve. The latter response factor represents the concentration at which the reference gives 50 % of the signal and can be calculated using a four-parameter (4-PL) logistic-log curve fitting model. The midpoint of the standard quantitation curve is a good indicator that the reference is still within its valid use date and is conveniently used to monitor continual assay performance over time.

In a first DOE, the influence of sample dilution scheme to working concentration, vortex duration, and "bench" hold time was studied. In Fig. 12.10, the vortex duration and the dilution scheme were found to significantly influence sample antigenicity (model p-value = 0.0047). A systematic error is found due to the dilution scheme while sample antigenicity is affected by longer vortex duration (greater than 5 s) due to change in antigen conformation and/or epitope accessibility. Protein aggregation related to shearing is known to affect antigenicity.

In a second DOE, different risk-assessed assay parameters were analyzed for their potential to impact sample antigenicity quantitation and assay quality performance, namely antigen load and concentrations of capture polyclonal antibody, detection monoclonal antibody "Ab2" and HRP-conjugated secondary antibody. As shown in Fig. 12.11, only the concentration of detection antibody "Ab2" was found to affect sample antigenicity although the statistical significance (p-value = 0.0138)

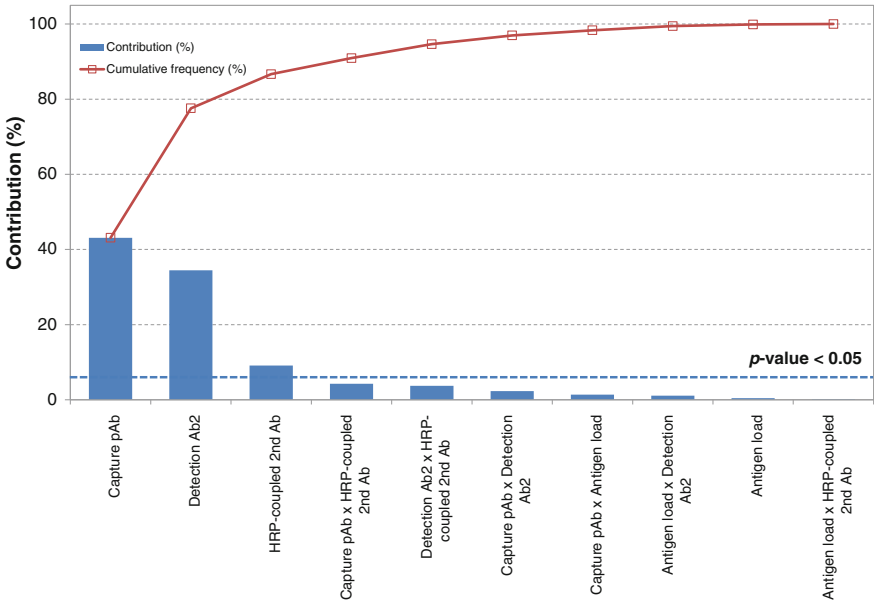


Fig. 12.12 Pareto chart of CMF impacting the midpoint of the reference

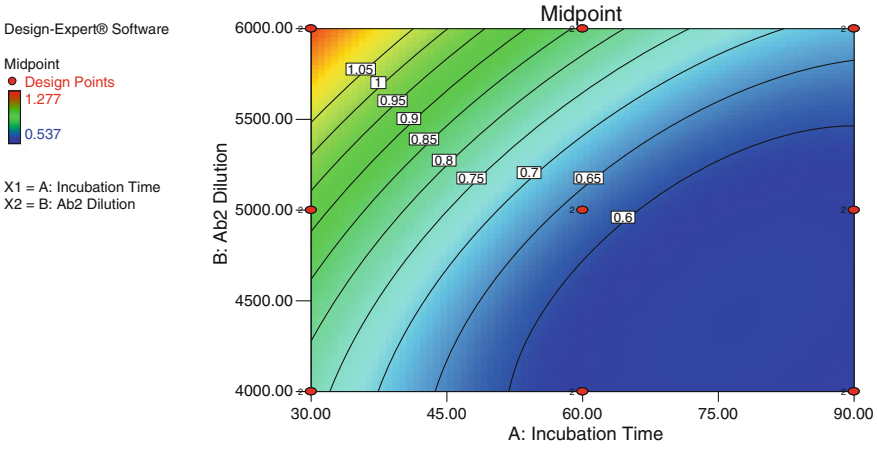


Fig. 12.13 3D contour plot of the reference midpoint as a function of Ab2 concentration and incubation duration

and relative impact are moderate with respect to concentration range (fourfold between lower and upper limits).

In addition to impact of CMF on sample quantitation, different parameters and second-order interactions were also shown to affect the midpoint absorbance of the

Table 12.10 Variance components and their contributions

Effect	Variance component	Percentage contribution (%)
HRP-coupled 2nd Ab	0.004497	58.9
Day	0.002809	36.8
Operator	0.000326	4.3
Buffer	0.000000	0.0
Total	0.007631	
%GCV	9.1 %	

reference. A Pareto chart is often useful in identifying what are the parameters and/or interactions with the highest order of magnitude (Fig. 12.12). Above a certain threshold, the parameters identified can be considered as a CMF.

For the CMF with the highest influence, a confirmatory set of experiments in the form of a response surface can be then used to confirm and decide on appropriate control strategies. Since the concentration of detection antibody "Ab2" was categorized as CMF, the response surface approach was used to evaluate the design space and boundaries of assay robustness. The "Ab2" incubation time at detection was selected for interaction. The response surface analysis suggests that a quadratic model is optimal. The contour plot (Fig. 12.13) is a convenient way to identify the design space and decide on potential acceptance criteria for reference suitability (e.g., OD > 0.8).

Finally, intermediate precision was assessed between laboratories, analysts, day-to-day testing, and different sample batches using a nested design. The choice of the design was based on random factors having the highest likelihood to impact assay variability, namely plate preparation, buffer preparation, operator, day, product batch, batch-to-batch variability of commercially available critical reagents. Independent sample preparations from two production batches were analyzed repeatedly using four plates performed on three different days by two analysts in parallel at each time ($N = 24$). One of the two batches used in the precision study corresponded to the lyophilized DP. Random factors such as buffer preparation and batch quality of the HRP-coupled secondary antibody were also included in the design to study their influence on the precision of the assay.

Total method variability was evaluated in addition to a variance component analysis to determine factors that impact method performance the greatest. The hierarchical design utilized a mix of prior knowledge as well as outcomes from the risk analysis to determine which factors to include and which replication strategy to employ. This allowed expressing precision results with associated confidence intervals displaying the level of uncertainty around communicated values.

Assay variability was expressed in geometric coefficient of variation (GCV in %) of log-normal data. Sources of random error, i.e., day, operator, buffer, HRP-coupled secondary antibody, were ranked according to their respective contributions to the total method variability. As shown in the Pareto chart, most of the

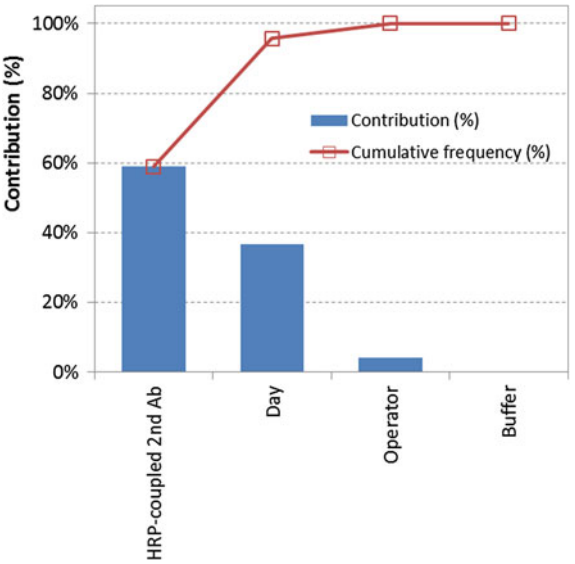


Fig. 12.14 Contributions to and cumulative frequency of variation

random variability is related to the change in the batch quality of HRP-coupled secondary antibody followed by the "Day" factor. Overall the method variability showed acceptable intermediate precision of 9.1 %. No further assay improvement was decided since intermediate precision was deemed acceptable for a potency assay (Table 12.10 and Fig. 12.14).

Other ICH characteristics were also confirmed using data from the intermediate precision including: the linearity and working range of the reference, suitability of the 4-parameter logistic regression model of the standard curve used for sample quantitation, as well as parallelism of the sample response and quantitation accuracy.

12.2.6 Method Control Strategy and Analytical Method Maintenance

Information on assay robustness and intermediate precision gathered for building the Design Space of the assay were then used to build a scientifically sound control strategy (Table 12.11). The Design Space of the assay being established, the understanding of the different factors impacting assay performance can support the design of appropriate control measures, including improvement of analytical instructions and clear documentation, setting of data-driven acceptance criteria and/or range for system/sample suitability or shelf-life of reagents and buffers.

Results from the intermediate precision study were used for qualifying an internal assay control and setting acceptance criteria for routine testing from the

Table 12.11 Summary of proposed method control strategy

Factor	Category	Impact	Control strategy
Dilution scheme	CMF	Bias/systematic error	Define clear instructions to perform sample dilution in the SOP
Vortex time	CMF	Sample accuracy	Define maximum vortex duration in the SOP, e.g. 3 s (<5 s)
Dilution of the detection Ab2	CMF	Sample accuracy	Define a target dilution in the SOP, e.g. 5000-fold, in combination with minimum incubation duration, e.g. 60 min
			Use control chart to monitor antigenicity of a qualified internal assay control
		Reference midpoint	Use control chart to monitor standard midpoint in routine
Batch quality of HRP-coupled 2nd Ab	Noise	Intermediate precision	Use control chart to monitor antigenicity of a qualified internal assay control
Day	Noise	Intermediate precision	Use control chart to monitor standard midpoint in routine

intermediate precision study. The lyophilized DP (averaged antigenicity from the precision study = 101 %) was selected with respect to its good stability profile. Acceptance criteria were defined as upper and lower limits of the prediction interval as follows (Eq. 12.1):

$$\text{Acceptance limits} = 101\ \% \pm t_{(95\,\%,N-1)} \times 9.1\ \% = [81\ \%; 121\ \%] \qquad (12.1)$$

Finally, method performance was monitored to ensure continual compliance toward established control measures. This was achieved by following test results from routine testing in statistical control charts as shown in the Fig. 12.15. Different parameters from the control strategy can be combined to trend for analytical outlier and initiate appropriate investigations on a timely manner. Shewhardt rules can be used to detect potential trends in control charts. The three-signal control chart limits displayed in Fig. 12.15 are most commonly used. Values beyond these control limits represent special cause variation, i.e., they are not caused by random fluctuations or noise. Special cause events should be investigated to determine root causes for continuous improvement and risk mitigation.

12.2.7 Case Study Summary

The analytical development was closed out by comparing the ATP requirements upfront and the associated CMAs with the results of analytical development (see Table 12.12). The assay was found to satisfy several CMAs relating to the ATP,

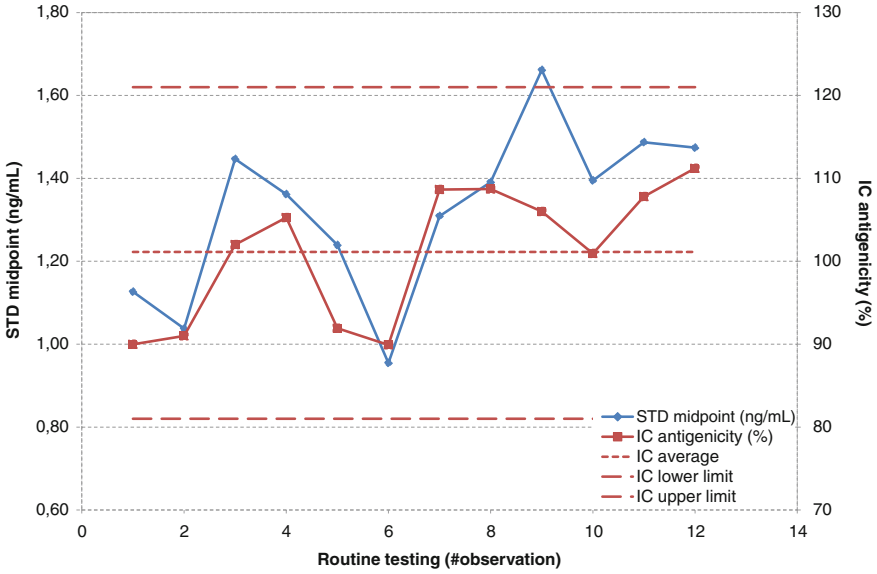


Fig. 12.15 Routine control chart for method monitoring

namely specificity, accuracy, and precision. Business-driven customer requirements were considered as well by establishing a method that was transferable, rugged, and robust.

12.2.8 Method Qualification and Validation

Method Qualification is the process of documenting an analytical method as suitable for its intended use for clinical manufacture or prior to the validation exercises for the final method used in commercial operation. Not all methods require validation but qualification may be expected in all other cases. There is currently no official guidance document for qualification of analytical methods, however, a valuable discussion is presented in (USP 2014) and a PDA task force is currently editing a Technical Report that encompasses analytical method development and qualification for biopharmaceuticals. Method Validation guidance is found elsewhere (Guidance for Industry 2008, 2009; PDA 2012).

Application of QbD for analytical methods is ideal as the basis for determining the limits within which the analytical method operates. This implies that the ATP becomes the basis for setting method qualification/validation acceptance criteria for the method parameters assessed in such studies (i.e., accuracy, precision, and specificity) that are directly linked to demonstrating suitability for intended use. In the case of method qualification with no protocol, the ATP criteria become the

Table 12.12 Summary of the case study

	ATP	CMA or KMA	Development outcome	Objective achieved
Quality target product profile	Potency for candidate vaccine antigen in accordance to ICH Q6B. Lot-release and stability potency testing of drug substance and drug product	Specific to native antigen	Potency assay specific to the product	+
		Decreased reactivity with degraded antigen		
Critical quality attribute	Antigen quality, 70–130% (provisional specification)	Accuracy, precision	≤30 % accurate	+
		Total error ± 30% within established working range		
		RSD_{IP} < 20 %		
Analytical method design and intent	State-of-the-art immunochemical quantification of antigen by	Throughput, Transferability	Double antibody sandwich ELISA	+
Release criteria	% antigenicity	Reportable value is antigenicity in % defined as the antigen-to-protein ratio	Measured raw data translated into % antigenicity scale	+
Development endpoint	Definition of factors during method design and establishment of instructions in an analytical procedure	Analytical procedure	Addressed high risks of assay conditions, reaction time, substrate incubation time and antibody dilution factor	+
Risk factors	Identification of critical factors using risk assessment and R&R (Intermediate precision and Robustness) experimental plan	Monoclonal detection antibody "Ab2" concentration	DOE of high ranking risk factors; design space of detection antibody and incubation time	+
Analytical method maintenance (not shown)	System suitability criteria, method robustness study	Control strategy	Specific examples of control charting (e.g., mid point of standard curve) and KM for this case study	+

Comparison of customer requirements as defined in the Analytical Target Profile (ATP) at start with achieved results and Critical Method Attributes (CMAs)

default acceptance criteria for the qualification exercise. This concept can be extended to the validation exercise as well. Robustness is a required method validation parameter but should be addressed in development (Guidance for Industry 2009). The DoEs used for robustness studies in the QbD approach are used to further demonstrate method suitability in addition to being useful for determining and then verifying system suitability limits.

Additionally the DoE approach used to assess intermediate precision in development can be used in the qualification/validation studies. The nested design approach is ideal for a typical intermediate precision validation study. This design analyzed using a linear mixed model with random effects has added benefit of determining the proportion of total variance attributed to each factor (e.g., day, operator, instrument) assessed in the design. Knowing the variance associated with each parameter gives information useful for future method troubleshooting or improvement. For example, in the case study, the IP data was analyzed using a linear mixed model with random effects. Three operators ran four plates each on 3 days (note in this dataset day and operator are confounded, therefore, only two factors were analyzed: operator/day and plate). The statistical output is given in Table 12.13.

The factors operator/day and plate contribute 11.5 and 88.5 %, respectively, to the total variability of the method under the design assessed. Future troubleshooting or improvement areas for the method could focus on these two factors.

12.3 Considerations Beyond Method Implementation

The systematic approach of QbD generates a large amount of knowledge around method design space, quality/business targets, risks, and their mitigation as well as potential opportunities for continuous improvement. To use these data most efficiently, knowledge generation, management, and transfer need to be well coordinated. Traditional approaches often separate development space from QC operations with transferred knowledge limited to the analytical procedure and some training in the form of qualification runs. With this approach, the vast amount of knowledge gained throughout development remains with assay developers, sometimes captured to a certain extent in technical reports but often forgotten between changing staff and time passing. Method risks and their mitigation rarely are captured and communicated systematically leading to avoidable assay failures far down-stream in operations.

Thus, QbD-based development needs to go hand-in-hand with systematic knowledge management as recognized to a certain extent in ICH Q10. The design space should be captured in technical reports detailing particularly knowledge around critical method parameters. However, data beyond the design space and around less-critical parameters should be conserved as well since it might be referenced at future time points, for example, when accommodating additional method transfers, reagent or equipment qualifications, or implementation of improvements.

Table 12.13 Variance components (LMM/REML) from intermediate precision data

Random effect	Var ratio	Var component	Std error	95 % lower	95 % upper	Pct of total
Op	0.1293281	7.9309028	24.358687	−39.81212	55.67393	*11.452*
Plate[Op]		61.323889	28.908358	29.013391	204.38348	*88.548*
Total		69.254792				100.000

Risks and their mitigation should be documented as well. This could be accommodated as a part of design space and development documentation, as a separate report focusing on the risk assessment tools, or combined with recommendations in the analytical procedure and control strategies. Similarly, control strategies may be incorporated into the procedure, for example, recommendations of specific equipment, or they could be documented as stand-alone documents, for example, reagent qualification plans that are key to the maintenance of immunoassays. These reports need to be transferred to the receiving laboratories together with the method to allow QC operations to effectively manage the method life-cycle. On the receiving end, knowledge retrieval and updates need to be managed as well. As QbD incorporates continuous improvement, it is imperative to incorporate experience gained during long-term method use both in the group carrying out the assays as well as the development laboratory which could incorporate the information into second generation assays or potential transfer knowledge to other projects as applicable.

In the early days of QbD, a number of discussions focused on the potential for regulatory flexibility around changes within the design space (according to ICH Q8 and Q11). However, few if any examples were realized in this direction although the filing and review process has been smoother when a QbD paradigm was followed. While filing a design space alone may still be in the future, the method understanding and risk knowledge gained throughout QbD-based development could help justifying partial validation approaches or comparability protocols, for example, when implementing method changes within the analytical method's design space. This will need to be managed on a case-by-case basis following quality systems together with using the risk assessment and mitigation process that is part of QbD. Overall, regulatory management of QbD may still be in its infancy.

Regardless of the regulatory component, scientific, and business advantages support QbD-based analytical development. Particularly, complex methods with multiple subprocesses such as immunoassays benefit from the systematic QbD development process compared to traditional approaches that rely heavily on individual's experience and line-of-sight.

References

Analytical Methods Committee, AMCTB No 55 (2013) Experimental design and optimization (4): Plackett-Burman designs. Anal Methods 5:1901–1903

Catania JP (2011) Quality by design: the case for change. BioPharm International, Duluth

CDER/CVM/ORA (2004) Guidance for industry: PAT—a framework for innovative pharmaceutical development, manufacturing, and quality assurance. U.S Food and Drug Administration: Rockville. http://www.fda.gov/downloads/Drugs/…/Guidances/ucm070305.pdf

Chen CW (2007) Regulatory flexibility through PAT and QbD—an FDA perspective. AAPS annual meeting, San Diego. http://mediaserver.aaps.org/meetings/07-AM/09-Reg-Relief/Chen.pdf

CMC Biotech Working Group (2009) A-Mab: a case study in bioprocess development. Version 2.1. http://www.ispe.org/pqli

CMC Vaccine Working Group (2010) A-Vax: applying quality by design to vaccines. Version 1.0. PDA

FDA (2004) Critical path initiative. http://www.fda.gov/ScienceResearch/SpecialTopics/CriticalPathInitiative/ucm076689.htm

Ferreira SLC, Bruns RE, Ferreira HS, Matos GD, David JM, Brandao GC, da Silva EGP, Portugal LA, dos Reis PS, Souza AS, dos Santos WNL (2007) Box-Behnken design: an alternative for the optimization of analytical methods. Analytica Chimica Acta 597(2):179–186

Guidance for Industry (2000) Analytical procedures and methods validation, chemistry, manufacturing, and controls documentation. US Food and Drug Administration, Rockville

Guidance for Industry (2006) Q9 Quality risk management. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER)

Guidance for Industry (2008) CGMP for phase 1 investigational drugs

Guidance for Industry (2009) Q8 (R2) Pharmaceutical development. U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER). ICH Revision 1, 25pp

Guidance for Industry (2011) Process validation: general principles and practices

ICH Harmonised Tripartite Guideline (2000) Good manufacturing practice guide for active pharmaceutical ingredients, Q7, current step 4 version, 10. In: International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use

ICH Q2 (2000) Analytical procedure and method validation guidance

ICH Q10 (2009) Pharmaceutical quality systems. U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER)

ICH Q11 (2012) Development and manufacturing of drug substances. ICH Q11 (2012) Development and manufacturing of drug substances. U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER)

Limentani GB, Ringo MC, Ye F, Bergquist ML, McSorley EO (2005) Beyond the t-test: statistical equivalence testing. Anal Chem 77(11):221A–226A

PDA (2012) Technical report 57: Analytical method validation and transfer for biotechnology products. Parenteral Drug Association

Ritter N, Advant SJ, Hennessey J, Simmerman H, McEntire J, Mire-Sluis A, Joneckis C (2004) What is test method qualification? In: Proceedings of the WCBP CMC strategy forum, 24 July 2003. BioProcess International, pp 2–10

Schweitzer M, Pohl M, Hanna-Brown M, Nethercote P, Borman P, Hansen G, Larew J (2010) Implications and opportunities of applying QbD principles to analytical measurements. Pharma Tech 34(2):52–59

USP 37-NF32 (2014) General Chapter <1033> Biological assay validation. United States Pharmacopeia. http://www.usp.org

Vander Heyden Y, Nijhuis A, Smeyers-Verbeke J, Vandeginste BGM, Massart DL (2001) Guidance for robustness/ruggedness tests in method validation. J Pharm Biomed Anal 24 (5–6):723–753

Chapter 13
Vaccine Potency Assays

Todd Ranheim, Ned Mozier and William Egan

13.1 Introduction

Historically, vaccines have predominantly been manufactured through complex biological processes (growth in embryonic chicken eggs, bacterial fermentation, and mammalian cell culture) that can be challenging to control and reproduce. Although the introduction of Quality by Design principles is changing the "the process is the product" mindset, the development of appropriate release assays remains a critical element in ensuring the safety and efficacy of a vaccine throughout its shelf life. The development of relevant and robust potency assays requires careful consideration of the nature of the protective immune response to the targeted antigen as well as a detailed understanding of the structural features of the antigen that elicit the protective response.

13.2 History

The concept of measuring the potency of vaccine formulations has been around nearly as long as the first vaccines. Shortly after the introduction of the smallpox vaccine in 1796, it was noted that cowpox, which was being transmitted from person to person, was declining in potency. The problem was solved by reintroducing the

T. Ranheim (✉)
Technical Development, Novartis Vaccines, Siena, Italy
e-mail: toddranheim@gmail.com; todd.ranheim@novartis.com

N. Mozier
Bioassays and Impurity Testing, Pfizer, St. Louis, MO, USA

W. Egan
Technical Development, Novartis Vaccines, Columbia, MD, USA

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6_13

virus back into cows and using serial inoculation of animals to maintain potency. This change to the then current practice had the added benefit of enabling large-scale production of the vaccine.

Potency as a concept was recognized, but the development of good methods to determine potency took quite a bit longer. It would not be until the late 1800s that local health authorities started introducing plans to monitor potency of biologics on a routine basis. And as late as 1902, practitioners were still lamenting the lack of a potency test for smallpox. "We unfortunately at present possess no test of the efficiency of lymph other than the clinical one… It is therefore of importance that, as is invariably done in the case of lymph issued from the Government Laboratories, every batch should be tested on children before being distributed for general use (Copeman 1902)."

Almost a century after the introduction of the vaccine, an animal potency assay for smallpox was developed by Calmette and Guerin (1901) and was further improved and standardized by Force and Leake (1927). The assay was improved yet again to reduce the variability inherent in an animal potency assay and was then ultimately replaced by an assay using titration onto chorioallantoic membranes of fertilized hen eggs and in cell culture (Kolb et al. 1961; Bartell and Tint 1961; Leparc-Goffart et al. 2003).

Several high-profile tragedies involving contaminated vaccines or antitoxins preceded the modern era of vaccine regulation and led to the passage in 1902 in the US of the Biologics Control Act. The Act was passed to "regulate the sale of viruses, serums, toxins, and analogous products" (Milstien 2004). The Act also created the Hygienic Laboratory of the U.S. Public Health Service, which in time became the National Institute of Health, whose mandate included providing oversight for the manufacture of biological drugs.

The twentieth century subsequently witnessed rapid advances in childhood immunizations as vaccines for rabies, pertussis, diphtheria, tetanus, polio, measles, rubella, mumps, Hib, hepatitis B, and meningitis among others were developed (CoPoP 2011; IAC 2006). Along with the development of these vaccines, the importance of manufacturing reproducibility and product quality testing became increasingly more recognized and subject to regulation by national authorities.

13.3 Definition and Link to Mechanism of Action

For the United States, potency is defined as the "… specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result (21CFR600.3 2013)." Potency tests are then defined such that they "… shall consist of either in vitro or in vivo tests, or both, which have been specifically designed for each product so as to indicate its potency

in a manner adequate to satisfy the interpretation of potency given by the definition in § 600.3 (s) of this chapter (21CFR610.10 2013).” The definition of potency is relatively straightforward, namely, the ability to effect a given result (for vaccines, the ability to produce an immune response that will protect against the indicated disease); the definition of potency tests is also straightforward—tests that indicate the vaccine’s ability to effect its intended result. The CFR allows potency tests to be either in vitro tests or in vivo tests, or both; importantly, the CFR does not require in vivo tests or bioassays for potency.

Other definitions for potency and potency tests, which differ somewhat from that in the CFR, exist. The ICH guidance, Q6B (Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products) (ICHQ6B 1999), notes that potency is “The measure of the biological activity using a suitably quantitative biological assay (also called potency assay or bioassay), based on the attribute of the product which is linked to the relevant biological properties.” This definition appears to disallow the sole use of chemical-based assays for measuring potency and, moreover, requires an assay that is based on the attribute of the product that is linked to its activity. The ICH Guidance, however, does exclude “conventional vaccines” from its scope.

The World Health Organization has adopted the ICH definition of Potency, e.g., in the WHO Guidance on the Non-clinical Evaluation of Vaccines (adopted by the ECBS in November, 2003) (WHO 2005), but has also noted in the same document that “Potency tests measure the biological activity of a vaccine but do not necessarily reflect the mechanism of protection in humans.”

Increasingly, less reliance is being placed on in vivo (animal-based) potency tests for vaccines, with more reliance being placed on in vitro-based assays (cell-based assays and binding assay), as well as purely chemical or physicochemical-based methods (such as those used for the polysaccharide–protein conjugate vaccines). As depicted in Fig. 13.1, quite commonly an inverse relationship exists between the

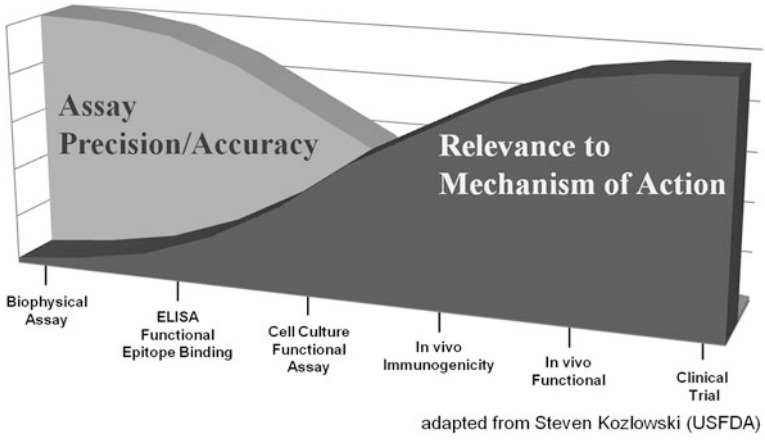


Fig. 13.1 Potency assay continuum

assay that is most representative of the desired biological effect and the assay that has the best statistical characteristics for use as a lot release and stability monitoring assay.

In Europe, an additional complication for the development and use of potency assays is a Directive to not use animal-based assays unless alternatives do not exist. Efforts are underway in Europe (and elsewhere) to replace animal-based potency tests that require a lethal challenge (such as the potency tests for rabies or anthrax) with either in vivo tests that do not require a lethal challenge or with in vitro tests (such as an ELISA-based method).

13.4 In Vivo Potency Assays

In vivo (animal-based) potency assays may be conveniently divided into two categories. Following inoculation with the test vaccine, the animals may either be challenged with virulent organisms (generally a defined reference strain of the homologous bacteria or viruses) against which the vaccine has been developed or, alternatively, the animal's immunological response is evaluated, e.g., by an ELISA or virus neutralization assay. An example of the former is the potency determination of rabies vaccine by the intracerebral challenge of mice with virulent rabies virus (Seligmann 1996); the potency is determined by the number of immunized mice surviving the challenge relative to the number of surviving non-immunized mice. The potency is calculated relative to surviving mice using a reference vaccine. An example of the latter is the potency determination of acellular pertussis vaccines by evaluating the serum antibody response in mice against the vaccine antigens using an ELISA-based assay (Ph.Eur.2.7.16 2012).

The variance of animal-based potency assays is generally large. For example, because of assay variance, the release criterion for the above-mentioned rabies potency test is 25–400 % of the reference. Additionally, because of the large variance in in vivo assays, the vaccine dose is often set by physical–chemical assays and the potency assay functions more as a limit test, i.e., given a particular dose of the vaccine, the potency test result exceeds a certain value. Additional concerns attend in vivo assays. They generally require considerable time to carry out, often 1–2 months, they are expensive, and importantly, they raise considerable ethical issues regarding the use of animals; with regard to this latter point, the European Union has issued a directive requiring the use of nonanimal-based assays whenever possible (EU 2010). Finally, for multiantigen vaccines, the substantial variance in the potency determination of each antigen may lead to the unwarranted rejection of potent lots of vaccine.

Given the many issues with in vivo assays, the question may be posed as to why they are used. The answer is complex. Many of the in vivo assays are associated with older vaccines (such as whole cells pertussis, diphtheria and tetanus toxoids,

rabies, anthrax), for which, at the time they were developed, other analytical technologies did not exist; additionally, the vaccines were often complex mixtures for which the protective constituents were not identified (e.g., whole-cell pertussis vaccine). Developing replacement in vitro assays that correlate with the in vivo assays has proven difficult, as has been seen in developing a replacement assay for rabies vaccine. For some of the more modern vaccines, the critical quality attributes for the various immunogens have been identified. To illustrate this point, we note that, for example, an ELISA assay may be used to determine the potency of hepatitis B vaccines because conformationally based protective epitopes have been identified on the protein antigen (Giffroy et al. 2006), similar results are not available for other vaccines, such as the acellular pertussis vaccines (although the dose of the acellular pertussis vaccines is set by the amount of each protein in vaccine, the potency is assessed by determining an immunological response in mice).

Currently a number of efforts are underway, both in Europe and the Americas, to find replacements or refinements for many of the older in vivo assays. As an example, the potency of diphtheria toxoids may be assessed by evaluating the serological response in guinea pigs to a test diphtheria vaccine in its ability to neutralize toxin in a Vero cell assay, rather than in a lethal toxin challenge to the immunized guinea pigs (Ph.Eu.2.7.6 2005). A number of efforts have been underway during the past decade to develop alternative potency assays for the rabies and anthrax vaccines, in great part because these assays involve lethal challenges to the inoculated and control animals. Efforts have also been underway —and in a number of instances have been successful—in reducing the number of animals that are needed for the potency test. For example, several assays that required testing of a set of dilutions have been replaced by single dilution assays, the diphtheria potency assay being one example. These efforts have been modeled on the three R ethical principles of Russell and Burch (Reduction, Refinement, and Replacement) (Russell and Burch 1959).

In vivo assays are often utilized during vaccine development. The in vivo assays provide a biological response to the candidate vaccine and a measure of the vaccine's potency that can be readily measured in the absence of a thorough characterization of the vaccine candidate or a knowledge of the vaccine's critical quality attributes. However, as the vaccine matures through the development cycle, in vitro assays are often developed and replace the in vivo assay. Such a development process is well illustrated by the development of Gardasil, Merck's human papilloma vaccine (Shank-Retzlaff et al. 2005). A mouse immunogenicity test was initially established and, during development and at the time of licensure, was replaced by an ELISA-based in vitro relative potency assay.

For currently licensed US vaccines, those employing an in vivo potency assay may be found in Table 13.1.

Table 13.1 Examples of US-licensed vaccine employing an in vivo potency determination

Vaccine or vaccine component	Potency test
Diphtheria toxoid[a]	Evaluation of serological response by animal challenge
Tetanus toxoids[a]	Evaluation of serological response by animal challenge
Inactivated polio virus (IPV)[a]	ELISA determination of serological response
Acellular pertussis[a]	ELISA determination of serological response
Rabies	Lethal challenge of immunized animals
Anthrax	Lethal challenge of immunized animals

[a] Applies also to combination vaccines containing this antigen

13.5 In Vitro Potency Assays

13.5.1 Live-Attenuated Viral and Bacterial Vaccines

The potency assay for the majority of live-attenuated viral vaccines has, in general, been a simple count of the number of infectious particles, primarily through a count of the number of plaque forming units. Potency assays for oral polio, mumps, measles, rubella, rotavirus, yellow fever, influenza, adenovirus, and smallpox all use methods to enumerate infectious particles.

A similar situation has existed for the live-attenuated bacterial vaccines, the *Salmonella typhi* vaccine (Ty21a) and the tuberculosis vaccines (BCG). The potency of these vaccines is determined by enumerating the number of colony forming units.

13.5.2 Inactivated Whole Viral Vaccines

Initially, the potency of the inactivated whole-virus vaccines was based on in vivo assays, namely, the immunological response to the vaccine in an animal species and the ability of the sera to neutralize the wild type virus or, as in the case of the rabies vaccine, the ability of the immunized animal to withstand a lethal challenge by wild type virus. Subsequently, in several instances, such assays have been replaced by in vitro assays designed to measure the quantity of a particular epitope on the vaccine virus as, for example, with the hepatitis A virus vaccine. The potency of inactivated whole-virus influenza vaccine is based on a single radial immune diffusion analysis.

13.5.3 Recombinant DNA Derived and Virus Derived Protein Vaccines

Examples of these vaccines include the hepatitis B virus and human papilloma virus vaccines. Although the original potency assays were in vivo assays (used either

initially after licensure or were initially used during vaccine development), the potency assays are currently ELISA-based assays that measure the quantity of particular epitopes on the proteins.

Other than the whole-virion inactivated vaccines, the inactivated influenza vaccines are either split vaccines or subunit vaccines. Their potency is measured by a SRID method.

13.5.4 Inactivated Bacterial Vaccines and Bacterial Protein Derived Vaccines

The potency of the whole-cell pertussis vaccine was measured by an intracerebral challenge of immunized mice with pertussis bacteria; this method remains the standard potency assay for those regions wherein whole-cell pertussis vaccines are in use. The potency of the current acellular pertussis vaccines are determined by monitoring the sera of immunized mice for antibody levels against the vaccine antigens using ELISA-based methods.

13.5.5 Bacterial Polysaccharide Vaccines

The potency of the bacterial polysaccharide vaccines (meningococcal, pneumococcal, and *Salmonella typhi* Vi antigen vaccines) is linked to the amount and size of the respective polysaccharides. The polysaccharide vaccines were the first vaccines whose potency was based on purely chemical and physical chemical methods. The potency may also include a measure of the content of certain appended groups to the polysaccharides, such as O-acetyl groups.

13.5.6 Bacterial Polysaccharide Protein Conjugate Vaccines

The potency of these vaccines is assayed primarily through chemical and physical chemical methods. The test parameters include polysaccharide content, free polysaccharide content, polysaccharide to protein ratio, degree of conjugation, and size (and polydispersity) of the conjugate. The primary measures of potency are the amount of saccharide conjugated to the protein carrier and the size/molecular weight of the conjugate.

13.5.7 Diphtheria and Tetanus Toxoids

The potency of these vaccines is based on the serological response to the vaccine in test animals. The response may be evaluated either by challenging the immunized animals to the toxin or by evaluating the ability of the immune sera to neutralize the toxin in an in vitro assay.

13.6 Method Development and Prevalidation

A rigorous period of method optimization is essential in the development of vaccine potency assays owing to their complexity and inherently high relative variability. Thoughtful consideration should be given to experimental designs that facilitate identification of all potential sources of variability (reagents, reference standards, time, temperatures, animal handling techniques, cell culture techniques and passage levels, seasonal factors, analysts, critical equipment, etc.) A potent approach to improving the quality of the final reportable result is to hardwire the assay acceptance criteria into the test method. The criteria for a "normal" assay should be built around the performance of a stable reference standard. By interrogation of the raw data from a stable reference standard tested repeatedly during the method development phase, analysts can establish statistically meaningful ranges for a variety of measurements including all the parameters of the curve fitting program, the precision of replicates, day to day variability, and others. This is also invaluable as a tool to assure that critical reagents (e.g., antibodies, binding ligands) are also not changing on storage or when replaced. These criteria can be most useful for objective decision-making for determining when a test is invalid, and before sample results are calculated.

13.7 Assay Validation

Validation of potency assays for vaccines is typically based on (1) the type of vaccine being tested and (2) the specific procedural details of the analytical method. The term "potency" is used differently across product class and within the vaccine class. For analytical procedures applied to vaccines, potency can mean the strength of the antigens or, more typically, the biological activity. There are examples of both, and this needs to be defined before test method validation is to be conceived. In all cases, knowledge and understanding of the active ingredient's intended human in vivo mode of action is the most useful information to develop before consideration of validation. Greater understanding may lead to more confidence in results, hence an expansion of options available to the manufacturer.

Options available to the method developer include assays that are based on animal, cell culture, biochemical (e.g., enzymic) and, in some cases, ligand and receptor binding. This range of options is broad enough for most vaccines and has been fully utilized in the field of vaccine potency assays owing to the great diversity of compounds. A number of similar approaches have been applied for vaccine products on the market as the history was developed well before the ICH guidelines were in effect. The applicability of ICH guidelines are less relevant to some products that have been in use for a long time, such as live or attenuated organisms; however, the concept of standardization and relative potency is well suited to the assessment of accuracy and precision which are defined in ICHQ2(R1) (1996). More commonly, vaccines that are purified (proteins, polypeptides, their derivatives, and conjugates produced recombinantly or non-recombinantly) appear to be within the scope of ICH 6B and vaccines. Vaccines that are "well-characterized proteins or peptides" are explicitly claimed in the scope of ICHQ5C (1995). Many modern vaccines bear great similarity to recombinant biotherapeutics in regards to what can be characterized. Taken together, the ICH quality documents are valuable for those developing and validating analytical methods and may be used by regulators in the USA, European Union, Japan, and other countries. For instance, ICHQ2(R1) (1996), though quite general to encompass small- and large-molecule drugs, clearly place potency assays in the category of Assay (henceforth assay and potency are used interchangeably). This is useful in that it specifies the data required for test method validation and establishes definitions for each characteristic measured during the exercise.

The types of materials (in process, intermediates, drug substance, drug product) for which test methods are validated depend on their intended use. Test methods which monitor the quality of these materials need appropriate validation to confirm materials are (1) adequate for further processing and (2) suitably stable during their period of intended use. Comprehensively, these tests must assure the final drug product is safe, pure, and efficacious. For this reason, emphasis is given on the validation of the potency assay test method for the final dosage form of the drug product. This activity is critical insofar as it represents the final measure of the potential efficacy of the drug as delivered to patients. Vaccine drug products have a unique challenge in that they may be combined with adjuvants or contain a mixture of antigens. In these cases, the assay must be able to individually quantitate each of the antigens, or the antigens can be separated from adjuvant (e.g., using suitable buffer treatments) without destruction of functional epitopes. If a suitable method is not found (either in vivo or in vitro), a strategy that shifts the emphasis to the validation of the assay method for the drug substance can be explored. Even in cases when the drug product is testable, manufacturers have an obligation to show that the drug substance maintains its potency over the shelf life and under the conditions of storage. In many cases, the drug substance and drug product may be validated together by diluting them to a matching antigen concentration and then testing identically.

In vivo potency assays are understood to have higher variability, except in rare cases where they are based on concentration of the active material, because of the

nature of the biological systems. Even the most genetically pure animal strains, housed and managed with great attention to consistency, will show differences between individuals. Adding to this is the endpoint readout, which has its own variation and may require the use of customized (manufacturer made and controlled) critical assay reagents (e.g., antibodies). These factors are well recognized and "wider confidence limits" (ICHQ6B 1999) are the norm. To minimize the variation from biological assays, a validated quantitative strength assays is an advantage since it enables close matching of the assay concentration of reference and test article. These sources of variation are the main factors leading to the relatively wide range of control limits and product specifications. In some cases, if the assay variation exceeds that of the process, the control limits will be based entirely on the assay capabilities. This underscores the value of a proper assay validation as a means to generate meaningful data as opposed to a box-checking exercise.

Historically, potency testing for vaccine drug products has been perceived as an opportunity and a challenge. The opportunity is that less testing (by physicochemical means) overall is possible if a credible, well standardized potency assay is available. However, the trend in vaccine development is clearly toward more characterization for product consistency regardless of the availability and quality of the potency assay. While the opportunity may be diminishing, the challenge is unchanged and predicated on the fact that a true mechanism-based mimic of how the vaccine works in the human patient is never possible. Unlike bioassays for other biotherapeutics, where the direct interaction of the active with the target or receptor (on cells, or binding after immobilization on a solid support) is possible, vaccines involve a complex cascade that is not replicated in any in vitro systems today. Vendors are working in this area, some with very clever approaches, but a credible replication of the human immune system to use for assay is currently not available. As a consequence, there are a great diversity of vaccine potency assays and the regulatory expectations are less predictable. A brief summary of the many approaches possible for the range of types of vaccine products, as described in the fine review by Hendriksen (2009), includes physicochemical methods (e.g., analytic, biochemical, spectroscopic); immunochemical (e.g., biosensor, ELISA, immunoblotting); in vitro immunological (antibody or cytokine production, lymphocyte activation/proliferation); or potency (e.g., functional antibodies [neutralizing, bactericidal], challenge tests). The latter category (challenge tests), though sometimes highly relevant as a mimic to function, is increasingly being avoided for ethical (animal welfare) and practical reasons.

The following sections are intended to summarize the various approaches taken for vaccine potency assay validation, but first the principles of validation according to ICH (ICHQ2(R1) 1996) are discussed as generally applicable to vaccines. Being mindful of the principles of validation and the reasoning for each type of data collected may simplify the process as applied to the exceedingly complex and diverse range of vaccine products. The simplest approach of all is in those rare cases where a physicochemical test is possible, in the manner in which small molecules rely on drug strength as the only measure of potency (inferring that all actives are

equally potent). The prerequisites for this approach, according to ICH Q6B, are that (1) sufficient physicochemical information including higher order structure is established and relevant correlation to biologic activity has been demonstrated; and (2) there exists a well-established manufacturing history. It is therefore incumbent on the manufacturer to collect this body of information and have enough convincing data to make this case at the time of registration and approval of the vaccine product. This approach is not discussed further, as it is rare and the requirements according to ICH (ICHQ2(R1) 1996) are straightforward.

Validation requirements for potency, defined as assay test methods in ICH (ICHQ2(R1) 1996), are that they are able to quantify the active moiety in samples. This is interpreted to mean that test results are reported numerically and that a control range is possible for the major component. The requirements for assay methods are accuracy, precision, specificity, linearity, and range. It is also suggested that robustness be addressed during method development after the procedure has been optimized. Ultimately the validation may be considered an evaluation of the test method itself and should expose any flaws, limitations or deficiencies. Therefore, it is prudent to conclude a base level of test method development well before embarking on the validation exercise. A noisy or non-specific assay cannot be solved by validation and may impact the control range possible and the eventually the quality of the dossier. Finally, revalidation may be required if the process or formulation changes. In these cases, careful bridging is important to maintain continuity of the product throughout development. This is generally achieved through judicious use of well-characterized reference standards.

Specificity is a primary aspect to confirm in potency method since the method should be able to differentiate among (1) closely related but undesirable structures that might be present at the time of manufacture or may form on storage and (2) impurities or formulation components that are present. This may be manifested in antibody-based assays that measure epitopes of interest (e.g., known or surmised to negatively impact the function of the vaccine in humans). It is recognized that "complete discrimination" is not always possible (ICHQ2(R1) 1996) and therefore other assay methods are needed. For example, the drug concentration (strength) is an assay to validate and compensate, as well as being used to match the concentrations of standard to the test article. Specificity may be established for the potency assay by showing that active (from drug substance) spiked into the final formulation (in drug product) gives equivalent results, i.e., a lack of interference. Specificity is especially valuable if potency is measured in samples from the process prior to final processing, where impurities may normally be present. In these cases, comparing the matched (active) concentrations of process samples to final drug substance in the potency assay will indicate if there is interference in the presence of impurities/processing components. Another way to confirm specificity is where a conjugate or toxoided vaccine is evaluated (using matched concentrations) at various stages of partial activation versus the final. A specific method will differentiate between partially activated (i.e., less active or inactive) and the intended product. If specificity is not established during method development, there is no reason to proceed

further with validation since the test method must be able to "see" as it is intended to measure and be "blind" to other substances that are not an active component.

Linearity for a potency assay is the least useful and most misinterpreted validation characteristic for the simple reason that most biological responses are not linear. This is acknowledged in ICH (ICHQ2(R1) 1996) where it is stated that immunoassays may not be linear and other approaches are allowed. In fact, regression analysis for potency assays are multifactorial, often four-parameter, and based on the nature of the data for a given assay. Regardless of the equation applied, the expectation is that at least 3, and preferably 5, concentrations will be used, an overall assessment of goodness of fit will be applied, and a data plot will be provided in the report. One approach, perhaps overlooked, is to simply transform the data by using the "calculated" Y values and X (known concentration) values to produce a linear response. In this manner, the required data for linear regression can be fulfilled (correlation coefficient, y-intercept, slope of the regression line, and residual sum of square). A well-characterized reference standard is typically used for this evaluation and this has practical value since the data produced for linearity is often the same replicated in routine application when the method is standardized and used routinely to test samples. In cases where a reference standard is utilized, it is expected that the responses of the test sample and the reference will be parallel.

Range may be determined from the bounded concentrations (upper and lower) of the standard dilutions tested for linearity (above). However, for potency assays, the most practical value is obtained by using (1) the suggested levels as a percentage bounding the target antigen concentration, (2) the range covered by any other physicochemical assay [e.g. strength], and (3) the shelf-life strength lower end of the acceptance range if applicable. ICH (ICHQ2(R1) 1996) recommends for assay that the target ±20 % should be studied as part of the validation. For instance, if the target vaccine concentration at release is 1.0 ug/mL, it should be evaluated at 0.8 and 1.2 ug/mL at a minimum. Using the same example, if the end of shelf specification is 0.7 ug/mL, this level should also be included. Ideally, a broader range to encompass most contingencies is desirable and is does not add to the burden if planned in the initial validation design. Range is unique among validation criteria, since it does not require data other than a listing of the range to be tested and the rationale. More importantly, the accuracy and precision is the data confirming the appropriate range. Note that if the study is designed with the appropriate number of concentrations and tests, including the target, the same data may be used for accuracy and precision without additional laboratory studies (see following on accuracy and precision).

Accuracy defined as the closeness of agreement between the value measured and that of a reference value. Since a reference value is obtained only through large, interlaboratory studies such as those sponsored by the USP or NIBSC, it is rarely applicable to new drugs under investigation. Several means to assess accuracy are described in ICH (ICHQ2(R1) 1996), the most pertinent being "comparison to an analyte of known purity," in most cases the manufacturer's reference standard. Also applicable is the fact that once specificity, linearity, and precision are established, accuracy is inferred. In practical terms, accuracy is measured by diluting a reference

Table 13.2 Example sample matrix for accuracy determination

		Preparation 1	Preparation 2	Preparation 3
0.7 mg/mL	Lower end of range	$X_{n\ =\ 1,\ 2\ \text{or}\ 3}^{a}$	$X_{n\ =\ 1,\ 2\ \text{or}\ 3}^{a}$	$X_{n\ =\ 1,\ 2\ \text{or}\ 3}^{a}$
0.8 mg/mL	80 % of target	$X_{n\ =\ 1,\ 2\ \text{or}\ 3}^{a}$	$X_{n\ =\ 1,\ 2\ \text{or}\ 3}^{a}$	$X_{n\ =\ 1,\ 2\ \text{or}\ 3}^{a}$
1.0 mg/mL	Target	$X_{n\ =\ 1,\ 2\ \text{or}\ 3}^{a}$	$X_{n\ =\ 1,\ 2\ \text{or}\ 3}^{a}$	$X_{n\ =\ 1,\ 2\ \text{or}\ 3}^{a}$
1.2 mg/mL	120 % of target	$X_{n\ =\ 1,\ 2\ \text{or}\ 3}^{a}$	$X_{n\ =\ 1,\ 2\ \text{or}\ 3}^{a}$	$X_{n\ =\ 1,\ 2\ \text{or}\ 3}^{a}$
1.3 mg/mL	Upper end of range	$X_{n\ =\ 1,\ 2\ \text{or}\ 3}^{a}$	$X_{n\ =\ 1,\ 2\ \text{or}\ 3}^{a}$	$X_{n\ =\ 1,\ 2\ \text{or}\ 3}^{a}$

[a] If assay tests samples as singlets ($n = 1$), 15 determinations required, if $n = 2$, 30 determinations required, if $n = 3$, 45 determinations required

material based on known ("actual") strength to exact concentrations, to include the target and a total of at least 3 levels that cover the specified range. Typically, 5 levels are tested including the least and greatest values in the range (above), plus the target (100 %) and 80 and 120 % of target. A total of 9 determinations are required, although it is often practical to test 15 (5 levels prepared independently in triplicate). This will result in 15 determinations and satisfy the requirement of at least 9. In addition, this will provide the data that can be used for precision (repeatability) below without additional laboratory study. Note that it is important that the intended protocol for the test method to be used routinely be followed exactly. For example, if the samples are to be analyzed in duplicate or triplicate and the results averaged, this should be included in the assay design. In this case, each of the 15 determinations would be tested twice (or thrice) after preparation and averaged to generate only 15 "reportable results." By this manner, the accuracy data will reflect the true method performance under conditions of actual use. An example is provided in Table 13.2 as a matrix for a vaccine where the target antigen concentration is 1.0 mg/mL and the necessary upper and lower ranges are 1.3 and 0.7, respectively.

For all determinations, the difference between the measured value and the actual is estimated by calculation of percent recovery. The confidence intervals are also required data.

Precision is the closeness of agreement (degree of scatter) between a series of measurements from sampling of a homogeneous sample. Precision is to be investigated in at least two ways: (1) repeatability (within assay) and (2) intermediate precision. A third way, reproducibility, is reserved for situations where the test method is to be performed in more than one laboratory, in which case it may be substituted for intermediate precision. *Repeatability* ("within" assay or intraassay) is typically performed by a single analyst under the same operating conditions over a short interval of time. It shows the capabilities of the method under the best possible conditions where samples are compared directly in the same test. *Intermediate precision* ("between" assay, within same laboratory or facility) is ideally performed by different analysts, on different days, using different instruments, and any other variables that analysts may be expected to encounter in the future. *Reproducibility* is the same as Intermediate precision but adds an additional variable, that of the laboratory. Intermediate precision and reproducibility information

is uniquely valuable in that it provides an estimate of variance that most mimics the application of the method in the future under all kinds of expected and random variables. For example, potency test results that seem out of trend in future stability studies can be interpreted as likely due to method versus a true change in the quality of the material. Care must be exercised to avoid validation experiments that are done in a short time under artificial control since it may suggest that the analytical test method has greater capabilities than are truly the case in normal use. For all types of precision, the required data is a determination of standard deviation, relative standard deviation (coefficient of variation), and confidence intervals.

Repeatability (Precision) minimally requires either (1) 6 determinations at target (100 %) of test concentrations or (2) 9 determinations of three concentrations across the range (each in triplicate). The latter approach allows assessment together with the accuracy determination (see above), where the standard deviation from the replicate values for each of the 5 levels may be calculated from the data.

Intermediate precision may use either the reference against itself or, ideally, another batch of material. Critical to this determination is careful design of the study so that normal variables (day of assay, analyst, instrument, etc.) are in play and represented in the final reportable result. These variables are necessarily confounded, but confined, since the purpose is to capture all normal variation in the reportable results. It should be noted that understanding of the major sources of assay variability are best studied during the method development phase, where they can be controlled and built into the test method prior to validation (see Robustness below). The validation exercise measuring intermediate precision is intended to give results as expected in the future under normal conditions of use. An example matrix is given in Table 13.3, where a single sample (separate from the reference standard) is tested by itself as a target (100 %) a total of 6 times, by two analysts in the same laboratory facility using two instruments. By this design, six reportable values are calculated for percentage relative potency. These results are averaged and standard deviation calculated. The percent relative standard deviation can then be used to calculate the statistical probability that a given result is different than 100 %, and future data interpreted as likely the same as reference or different. The minimal number of data points is not specified in ICH (ICHQ2(R1) 1996), but at least three results are necessary to calculate standard deviation, and at least two analysts for at least 2 days are necessary to meet the minimum design requirements.

Table 13.3 Example sample matrix for intermediate precision determination

	Analyst 1	Analyst 2
Day 1	Instrument 1	
Day 2	Instrument 2	
Day 3	Instrument 2	
Day 4		Instrument 2
Day 5		Instrument 1
Day 6		Instrument 1

Reproducibility may be required if more than one laboratory will be performing the potency assay. In this case, the matrix above may be modified such that Analyst 2 is from the second laboratory. In this case, two instruments in the same lab may not be required because the separate laboratories will have their own units. If intermediate precision was completed as per the design above, and a new laboratory is being qualified, reproducibility can be conducted by simply having the new facility execute the same design as one of the analyst using identical samples (stably stored so there is no degradation). The dataset will then increase from six reportable results to nine, and the performance in the new facility can be directly compared to the originating laboratory. This is a practical and economical approach, but should not be conducted until the receiving laboratory has been trained and become familiar with the method.

Robustness is recommended to be conducted during method development and is an evaluation of the influence of assay conditions deliberately manipulated to understand their impact on results. It is not to be confused with intermediate precision (normal variation under typical conditions of use). Robustness is therefore an exercise to understand the susceptibility of the test method to known variations. It is expected that those parameters critical to performance will then be controlled for in future executions of the test method. For example, if an incubation step is thought (or known) to be susceptible to the concentration of antibody, then deliberately prepared antibody dilutions will be tested against each other and a range (or maximum dilution) written into the test method. No step in an analytical method should knowingly be near the edge of failure and, if necessary, should be controlled with training and special precautions written into the test method procedure.

13.8 Stability

The stability of vaccine preparations during their period of use is an essential aspect to confirm during clinical development and before commercialization. The first requirement of a robust stability system is to establish that the potency method itself is stability indicating. This is typically established by treating samples with the mildest possible conditions to cause structural changes in the antigens. This may require experimentation with a range of antigen concentrations, buffers, pH, temperature, and all against time. A simple protocol for a vaccine to be stored at 4 °C is to test an accelerated (e.g., 25 °C) and stressed (e.g., 37 °C) condition for a period of 2–4 weeks. As these conditions are relatively mild, if the potency is diminished compared to untreated controls, these may be ideal for generating evidence that the potency assay is stability indicating under conditions where samples might be inadvertently exposed during shipping. Other characterization is necessary to understand which structural elements influence the potency result, although caution is advised since treatments can cause a multiplicity of alterations, any one or combination thereof altering potency. In some cases, the potency assay may be more sensitive than any other technique in detecting the structural change, in which case

its use is increasingly vital to product understanding. If the relatively mild conditions are ineffective in changing the potency, higher temperatures and harsher conditions may be employed. It is important to include a range of characterization methods in addition to the potency method, (including the method for strength) to confirm that the potency is altered. Otherwise, the strength measurement alone would be sufficient for potency. In cases where there is no other potency test possible, monitoring strength (drug concentration) as a function of storage time may be sufficient.

The ability to trend potency assay data as a function of time during stability studies is made challenging for assays with high variability. For this reason, a replication strategy should be used to increase confidence in the mean or "true" value. Of course, the cost due of replication can be very high (in materials and animals) and needs to be considered before embarking on stability studies. It is important to include the potency test for the final time points in stability studies in order to ensure that shelf life is well established for the vaccine drug product as used clinically or commercially. The stability of drug substance is important early to support preclinical studies to establish that there are no changes during the time it is stored prior to drug product manufacturing.

13.9 Reference Standards

A reference vaccine preparation is a critical component for many potency assays and is used to control for experimental factors that can vary over time and to provide a link between a vaccine preparation that was effective in the clinic and subsequent commercial lots. The World Health Organization maintains guidelines for the establishment and characterization of reference standards (WHO 2006). The goal of the WHO program is to establish international reference standards that allow the activity of biological products to be expressed the same way by various laboratories throughout the world and thereby provide a consistent basis for measurement and comparison. The WHO reference standards are typically provided in small quantities that are intended to be used to calibrate secondary standards that are prepared and routinely used by each laboratory (WHO 2014).

For novel vaccines where an international reference standard is not available, the WHO guideline provides a blueprint for the establishment of an internal reference standard, its use, and maintenance. Of particular note, a reference standard should resemble as closely as possible the response and characteristics of the test samples in the specific potency assay used. "The general principle is that of "like versus like." Thus although it may not be necessary for the standard to be prepared in the same formulation or matrix as test samples, it is necessary that the dose–response characteristics of the standard are the same as those of tests samples. In addition, the establishment of primary and secondary tiers of reference standards is highly recommended. The primary standard functioning as a "gold standard" against which all future secondary standards are calibrated to avoid the propagation of errors that can occur when standards are sequentially calibrated.

13.10 Potency Assay Challenges

The development of a novel potency assay can present significant technical and scientific challenges. These challenges can be compounded by many factors, including properties of the vaccine active ingredient(s) and/or the excipients added during formulation. The development of new or modified assays for older vaccines also presents unique obstacles.

13.10.1 Combination Vaccines

The development of combination vaccines has been encouraged in the drive to reduce the number of immunizations children receive and to increase efficacy by incorporating additional antigens. With the addition of each new antigen, the development of a potency assay becomes correspondingly much more complex. Current regulatory expectations assume that the potency of each of the individual components of a vaccine will be determined in the final container. Therefore, the first question to be answered is whether a single assay can be sufficient for all the components or whether several distinct assays will be required (e.g., DTaP potency assays). It must be determined if the presence of one or more of the components interferes with or introduces a bias in the measurement of any of the other components.

13.10.2 Adjuvants

Many vaccine preparations include adjuvants that are used to boost the immune response to the target antigen(s). While adjuvants can be critical to the efficacy of vaccines, they also can present unique challenges in the context of in vitro potency assays. (In vivo potency assays are typically performed in the presence of the adjuvant). The presence of adjuvants, such as aluminum salts, can interfere with the detection of the target antigen. Several vaccine potency assays have circumvented this problem by desorbing the antigen from the adjuvant prior to analysis (Shank-Retzlaff et al. 2005; Coombes et al. 2009; Shanmugham et al. 2010). The stability of the interaction of the antigen and adjuvant over time should also be carefully studied and understood in the context of the potency assay.

Potency assays for vaccines come in diverse forms and vary greatly in terms of format, complexity, statistical characteristics, and their ability to predict the therapeutic effect of the target antigen(s) (Table 13.4). Technological advances and quality by design principles will continue to change the way companies and regulators approach these assays and the subsequent improvement in understanding critical quality attributes will lead to better monitoring of vaccines at release and through shelf life. The tools and approaches may continue to evolve but the central and critical role potency assays play in vaccine analysis will persist into the foreseeable future.

Table 13.4 List of approved vaccines with selected potency assay references

Organism	Vaccine	Company	Potency reference[a]
Live virus			
Smallpox	ACAM2000	Acambis	Bartell and Tint (1961), Kolb et al. (1961), Leparc-Goffart et al. (2003)
Measles/Mumps/ Rubella	M-M-R II (MMR)	Merck	Schalk et al. (2005)
	ProQuad (MMRV)	Merck	
Chickenpox	Varivax	Merck	Van Vliet et al. (1987)
	ProQuad (MMRV)	Merck	
	Zostavax	Merck	
Rotavirus	RotaTeq (RV5)	Merck	Ranheim et al. (2006)
	Rotarix (RV1)	GSK	
Influenza	FluMist (LAIV)	MedImmune	Mo et al. (2008)
Yellow fever	YF-Vax	Sanofi	Monath et al. (2010)
Inactivated virus			
Polio	Ipol	Sanofi	Souvras et al. (1980)
	Poliovax	Sanofi	
Japanese encephalitis	Ixiaro	Novartis	Kikukawa et al. (2012)
	JE-CV	Sanofi	
Hepatitis A	Havrix (HepA)	GSK	Poirier et al. (2000)
	Vaqta (HepA)	Merck	
	Epaxal (HepA)	Crucell	
	Twinrix (HepA-HepB)	GSK	
Influenza (seasonal)	Afluria	CSL	Wood et al. (1977)
	Agriflu	Novartis	
	FluLaval	GSK	
	Fluarix	GSK	
	Fluvirin	Novartis	
	Flucelvax	Novartis	
	Fluzone	Sanofi	
	Flublok	Protein Sci.	
Rabies	Imovax	Sanofi	(Seligmann 1996)
	RabAvert	Novartis	
Viral proteins and VLPs			
Hepatitis B	Twinrix (HepA-HepB)	GSK	Stephenne (1990), Emini et al. (1986)
	Pediarix (DTaP-HepB-IPV)	GSK	
	Engerix-B (HepB)	GSK	
	Recombivax HB	Merck	
Human papillomavirus	Gardasil (HPV4)	Merck	Shank-Retzlaff et al. (2005)
	Cervarix (HPV2)	GSK	

(continued)

Table 13.4 (continued)

Organism	Vaccine	Company	Potency reference[a]
Live bacteria			
Tuberculosis	BCG	Merck	Smith et al. (1979)
	Tice BCG	Merck	
	Mycobax	Sanofi	
Typhoid	Vivotif	Crucell	
Toxoid			
Pertussis/Tetanus/ Diptheria	Decavac (Td)	Sanofi	Ph.Eur.2.7.16 (2012), Ph.Eu.2.7.6 (2005), Coombes et al. (2012), Sesardic (2012)
	Daptacel (DTaP)	Sanofi	
	Infanrix (DTaP)	GSK	
	Boostrix (Tdap)	GSK	
	Adacel (Tdap)	Sanofi	
	Tripedia (DTaP)	Sanofi	
	Kinrix (DTaP-IPV)	GSK	
	Pediarix (DTaP-HepB-IPV)	GSK	
	Pentacel (DTaP-IPV/Hib)	Sanofi	
Anthrax	Biothrax	Emergent	Hering et al. (2004)
Polysaccharide			
Pneumococcal	Prevnar13 (PCV13)	Pfizer	Kim et al. (2003)
	Pneumovax 23 (PPSV23)	Merck	
Typhoid	Typhim Vi	Sanofi	
Haemophilus type B	ActHIB (Hib)	Sanofi	Habig (1993)
	PedvaxHIB (Hib)	Merck	
	Hiberix (Hib)	GSK	
	Comvax (Hib-HepB)	Merck	
	Pentacel (DTaP-IPV/Hib)	Sanofi	
Meningococcal	Menactra (MCV4)	Sanofi	Habig (1993)
	Menomune (MPSV4)	Sanofi	
	Menveo (ACWY)	Novartis	
	Bexsero (B)	Novartis	
	MenHibrix (C,Y,Hib,T)	GSK	

[a] Vaccine package inserts typically contain basic information about the potency assay. Package inserts for vaccines licensed in the US can be found on the FDA website (FDA 2013)

References

21CFR600.3 (2013). CFR title 21 600.3. FDA.gov: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=600.3. Accessed 7 April 2014

21CFR610.10 (2013). CFR title 21 610.10. FDA.gov: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=610.10. Accessed 7 April 2014

Bartell P, Tint H (1961) Correlation of three potency assay methods for smallpox vaccines. J Immunol 348–353

Calmette A, Guerin C (1901) Recherches sur la vaccine experimentale. Ann Inst Pasteur 15:161

Coombes L, Stickings P, Tierney R, Rigsby P, Sesardic D (2009) Development and use of a novel in vitro assay for testing of diphtheria toxoid in combination vaccines. J Immunol Met 350 (1–2):142–149

Coombes L, Tierney R, Rigsby P, Sesardic D (2012) In vitro antigen ELISA for quality control of tetanus vaccines. Biologicals 40(6):466–472

Copeman SM (1902) Modern methods of vaccination and their scientific basis. Med Chir Trans 85:243–281

CoPoP (2011) Timelines. From the history of vaccines: http://www.historyofvaccines.org/content/timelines/all. Accessed 18 April 2014

Emini EA, Ellis RW, Miller WJ, McAleer WJ, Scolnick EM, Gerety RJ (1986) Production and immunological analysis of recombinant hepatitis B vaccine. J Infect 13(Suppl A):3–9

EU (2010). Legislation for the protection of animals used for scientific purposes. European Commission: http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm. Accessed 18 April 2014

FDA (2013) Approved vaccines. FDA.gov: http://www.fda.gov/biologicsbloodvaccines/vaccines/approvedproducts/ucm093830.htm. Accessed 24 May 2014

Giffroy D, Mazy C, Duchene M (2006) Validation of a new ELISA method for in vitro potency assay of hepatitis B-containing vaccines. Pharmeuropa Bio 2006(1):7–14

Habig WH (1993) Potency testing of bacterial vaccines for human use. Vet Microbiol 37:343–351

Hendriksen C (2009) Replacement, reduction and refinement alternatives to animal use in vaccine potency measurement. Expert Rev Vaccines 8(3):313–322

Hering D, Thompson W, Hewetson J, Little S, Norris S, Pace-Templeton J (2004) Validation of the anthrax lethal toxin neutralization assay. Biologicals 32:17–27

IAC (2006) Immunization action coalition. Vaccine Timeline: http://www.immunize.org/timeline/. Accessed 18 April 2014

ICHQ2(R1) (1996) Validation of analytical procedures: text and methodology. ICH.org: http://www.ich.org/products/guidelines/quality/quality-single/article/validation-of-analytical-procedures-text-and-methodology.html. Accessed 9 April 2014

ICHQ5C (1995) ICH Q5C Stability testing of biotechnological/biological products. ICH.org: http://www.ich.org/products/guidelines/quality/quality-single/article/stability-testing-of-biotechnologicalbiological-products.html. Accessed 9 April 2014

ICHQ6B (1999) ICH Q6B specifications: test procedures and acceptance criteria for biotechnological/biological products. ICH.org: http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html. Accessed 8 April 2014

Kikukawa A, Gomi Y, Akechi M, Onishi T, Manabe S, Namazue J et al (2012) Superior immunogenicity of a freeze-dried, cell culture-derived Japanese encephalitis vaccine (inactivated). Vaccine 30:2329–2335

Kim KH, Yu J, Nahm MH (2003) Efficiency of a pneumococcal opsonophagocytic killing assay improved by multiplexing and by coloring colonies. Clin Vaccine Immunol 10:616–621

Kolb RW, Cutchins EC, Jones WP, Aylor HT (1961) A comparison of the rabbit scarification technique with titrations in cell cultures for the potency assay of smallpox vaccine. Bull World Health Org **25:**25–32

Leake JP, Force JN (1927) A method for estimating the potency of smallpox vaccine. Bulletin of the hygienic laboratory, No. 149. U.S.P.H.S, Washington

Leparc-Goffart I, Poirier B, El Zaouk A, Tissier M-H, Fuchs F (2003) New generation of cell culture assay for smallpox vaccine potency. J Clin Microbiol 41(8):3687–3689
Milstien JB (2004) Regulation of vaccines: strengthening the science base. J Public Health Policy 25(2):173–189
Mo C, Yamagata R, Pan A, Reddy J, Hazari N, Duke G (2008) Development of a high-throughput Alamar blue assay for the determination of influenza virus infectious dose, serum antivirus neutralization titer and virus ca/ts phenotype. J Virol Met 150:63–69
Monath TP, Lee CK, Julander JG, Brown A, Beasley DW, Watts DM et al (2010) Inactivated yellow fever 17D vaccine: development and nonclinical safety, immunogenicity and protective activity. Vaccine 28:3827–3840
Ph.Eu.2.7.6 (2005) European pharmacopoeia 8th edition—2.7.6. EDQM.eu: http://www.edqm.eu/en/european-pharmacopoeia-8th-edition-1563.html. Accessed 18 April 2014
Ph.Eur.2.7.16 (2012) European pharmacopoeia 8th edition—2.7.16. EDQM.EU: http://www.edqm.eu/en/european-pharmacopoeia-8th-edition-1563.html. Accessed 9 April 2014
Poirier B, Morgeaux S, Variot P, Fuchs F (2000) In vitro potency assay for hepatitis A vaccines. Biologicals 28(4):247–256
Ranheim T, Mathis P, Joelsson D, Smith M, Campbell K, Lucas G et al (2006) Development and application of a quantitative RT-PCR potency assay for a pentavalent rotavirus vaccine (RotaTeq®). J Virol Met 193–201
Russell W, Burch R (1959) The principles of humane experimental technique. Methuen, London
Schalk J, de Vries C, Jongen P (2005) Potency estimation of measles, mumps and rubella trivalent vaccines with quantitative PCR infectivity assay. Biologicals 33(2):71–79
Seligmann EB (1996) The NIH test for potency. In: Meslin F, Kaplan M, Koprowski H (eds) Laboratory techniques in rabies. WHO, Geneva
Sesardic T (2012) Bioassays for evaluation of medical products derived from bacterial toxins. Curr Opin Microbiol 15:310–316
Shank-Retzlaff M, Wang F, Morley T, Anderson C, Hamm M, Brown M et al (2005) Correlation between mouse potency and in vitro relative potency for human papillomavirus type 16 virus-like particles and Gardasil® vaccine samples. Human Vaccines 1(5):191–197
Shanmugham R, Thirumeni N, Rao VS, Pitta V, Kasthuri S, Singanallur NB et al (2010) Immunocapture enzyme-linked immunosorbent assay for assessment of in vitro potency of recombinant hepatitis B vaccines. Clin Vaccine Immunol 17(8):1252–1260
Smith D, Harding G, Chan J, Edwards M, Hank J, Muller D et al (1979) Potency of 10 BCG vaccines as evaluated by their influence on the bacillemic phase of experimental airborne tuberculosis in guinea-pigs. J Biol Stand 7:179–197
Souvras M, Montagnon B, Fanget B, van Wezel AL, Hazendonk AG (1980) Direct enzyme linked immunosorbent assay (ELISA) for quantification of poliomyelitis virus D-antigen. Dev Biol Stand 46:197–202
Stephenne J (1990) Development and production aspects of a recombinant yeast-derived hepatitis B vaccine. Vaccine 8(Suppl):S69–S73
Van Vliet JH, Colinet G, Yane F, Lemoine P (1987) A simplified plaque assay for varicella vaccine. J Virol Met 18(2–3):113–120
WHO (2005) Biologicals. WHO: http://www.who.int/biologicals/publications/trs/areas/vaccines/nonclinical_evaluation/en/. Accessed 8 April 2014
WHO (2006) Reference standards. WHO: http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf?ua=1. Accessed 2 May 2014
WHO (2014). Catalogue. WHO: http://www.who.int/bloodproducts/catalogue/Vacc2014.pdf?ua=1. Accessed 1 May 2014
Wood JM, Schild GC, Newman RW, Seagroatt V (1977) An improved single-radial-immuno-diffusion technique for the assay of influenza haemagglutinin antigen: application for potency determinations of inactivated whole virus and subunit vaccines. J Biol Stand 5:237–247

Chapter 14
Establishing a Shelf Life and Setting Lot-Release Specifications

William Egan and Timothy Schofield

14.1 Introduction

Potency is a critical quality attribute of a vaccine and those attributes that impact potency are essential to assuring vaccine quality. Over its entire shelf life, a vaccine must exceed a minimal potency value and, when defined, cannot exceed a maximal potency.[1] Thus, over their shelf lives, vaccines must remain either above a minimum value of potency or within a defined range of potency. The upper and lower potency limits are established through clinical studies, with the lower limit based on efficacy and the upper limit based on known or potential safety concerns. As illustrated in Fig. 14.1, the vaccine's potency at release (expressed as a $TCID_{50}$ value in the Figure for a hypothetical live attenuated viral vaccine) and its associated shelf life are linked by the rate at which the vaccine, under defined storage conditions, loses potency. Thus, for the hypothetical viral vaccine in Fig. 14.1, with a lower allowed potency limit of 3,000 $TCID_{50}$s, its effective shelf life is nearly 36 months if released at 5,000 $TCID_{50}$s or *ca*, 19 months if released at 4,000 $TCID_{50}$s. The choice of the shelf life and its associated release value would be the manufacturer's decision (assuming that both release values were shown to be safe). In establishing a release potency value and shelf life specification, the uncertainties in the potency determination at release and rate of potency loss must also be

[1] Although maximal potency values are set for the majority of vaccines, certain vaccines have no defined upper potency limit.

W. Egan (✉)
Novartis Vaccines and Diagnostics, 350 Massachusetts Avenue,
Cambridge, MA 02139, USA
e-mail: William.egan@novartis.com

T. Schofield
MedImmune, One MedImmune Way, Gaithersburg, MD 20878, USA
e-mail: SchofieldT@MedImmune.com

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6_14

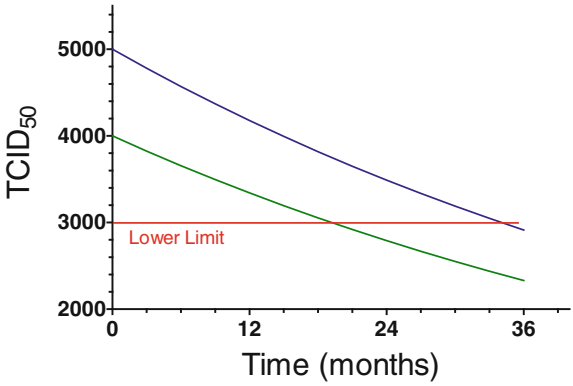


Fig. 14.1 Time dependence of the potency value for a hypothetical vaccine that would be released at either 5,000 or 4,000 $TCID_{50}$ units

established and accounted for; these uncertainties are not illustrated in Fig. 14.1. This chapter will focus on the determination of a vaccine shelf life and release specification, including the effects of statistical uncertainties using potency as an example attribute.

14.2 Establishing the Release Specification and Shelf Life

14.2.1 Establishing the Rate of Loss of Potency

Consider a hypothetical live attenuated viral vaccine and its associated potency values ($TCID_{50}$ values; herein expressed as their natural logarithm) as a function of time for three vaccine Lots as depicted in Fig. 14.2 (the $TCID_{50}$ values depicted in Fig. 14.2 are presented in Appendix). The immediate question is how to use these three data sets to establish a shelf life and associated release specification for the

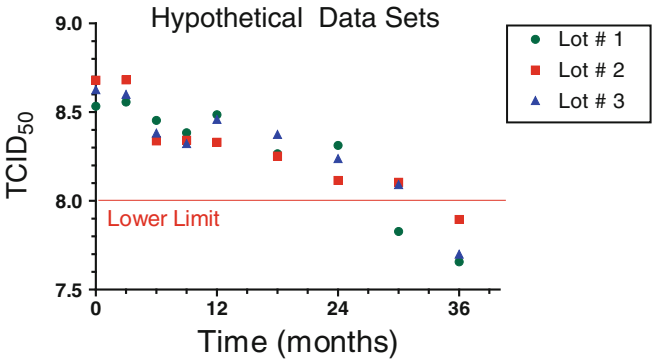


Fig. 14.2 Simulated time dependence of the potency ($TCID_{50}$ values; expressed as their natural logarithm) for three Lots of a hypothetical live attenuated viral vaccine

vaccine. Most simply, it could be concluded that if the vaccine were released at 5,000 $TCID_{50}$s [ln(5000) ≈ 8.52], or more, as permitted, then the vaccine's potency would remain within specification, i.e., above 3,000 $TCID_{50}$s [ln(3000) ≈ 8.0], for 24 months (although two of the Lots remained above this lower limit at 30 months, this was not the case for Lot #1 which fell below the lower limit at the 30-month time point); the shelf life could be set as 24 months, the last time point for which all three Lots remained within specification.

The above-described model for setting a shelf life, which may be termed a "compliance model" as it sets a shelf life by the length of time that the vaccine's potency measurements remain above (are compliant with) the assigned lower limit, while workable and having the advantage of simplicity, suffers several drawbacks.[2] First, there is an overreliance placed on the one or few data points that appear outside of the specification window; moreover, the totality of stability information that is contained within the other data points, namely, how potency is changing with time, is ignored. Second, there is a realization that increasing the number of Lots (or the number of time points for a given lot) that are tested will result in an increased probability that a test result, due simply to the random errors inherent in the assay, will fall outside the specification window and, therefore, result in a shortened shelf life. This discourages the collection of additional data. Third, a sense of the confidence or extent of certainty that may be placed in the shelf life is lacking. Finally, the shelf life of the vaccine would be undefined if the vaccine were to be released at a lower potency value.

As an alternative procedure, and to overcome the above-noted deficits, the time and potency data sets may be fit to a mathematical model (regression analysis) to obtain an estimate of the time dependence of the potency loss. The regression analysis would utilize all of the data and not rely on a single point or select set of data points. From the fitting process, an estimate for the mathematical model as well as a measure of the confidence in that estimate, expressed as a standard deviation or confidence interval on the regression line, is obtained. Non-mechanistically associated mathematical models, for example, fitting the data to a polynomial equation, or mechanistically associated models, such as a particular chemical kinetic model (with its accompanying mathematical form), can be explored.

Changes in vaccine potency arise from various processes. For a protein-based vaccine, the changes may derive from denaturation/aggregation, hydrolysis, deamidation, oxidation, disulfide interchanges, or other transformations to which proteins are subject. For a polysaccharide-based vaccine (including polysaccharide-protein conjugates), the changes in potency may derive, inter alia, from hydrolysis of the saccharide main chain or side chain, or loss of particular appended groupings (such as O-acetyl or pyruvate). For a live attenuated virus vaccine, changes in

[2] The World Health Organization, in its Guidelines on Stability Evaluation of Vaccines, has noted that "In many countries, expiry periods of vaccine products are calculated by testing a predefined number of Lots, at pre-defined intervals, and designating the expiry period as the first time at which a stability measurement falls below an acceptable threshold". The WHO Guideline also notes that "This approach has the advantage of simplicity, but may yield spurious results due to assay variability".

potency may derive from various changes which inhibit the ability of the virus to enter cells or to replicate within those cells. Such changes may be modeled—and have been modeled—as processes following particular kinetic rate equations. Oftentimes, these changes in potency, typically losses in potency, may be modeled as a first-order reaction, i.e., as a single exponential process. However, at times, more complicated kinetic models (for example, biphasic kinetics) are necessary. In this chapter, we will illustrate general concepts, treating changes in potency as simple exponential processes, i.e., as first-order kinetic processes.

14.2.1.1 First-Order Kinetics

The simplest plausible model for the loss of vaccine potency would be based on first-order kinetics, where the potency of the vaccine follows a rate law of the form

$$P(t) = P(0)e^{-kt} \tag{14.1}$$

where,

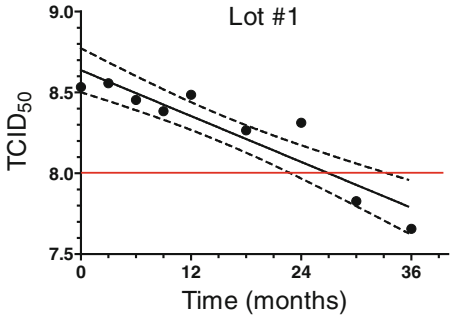
$P(t)$ is the vaccine potency at time, t
$P(0)$ is the vaccine potency at time zero
k is the rate constant for the loss of potency
e is the base of the natural logarithm

In Eq. 14.1, $P(0)$ is the time that the kinetic measurements are begun. Equation 14.1 may be linearized by taking the natural logarithm of both sides of the equation. Thus,

$$\ln P(t) = \ln P(0) - kt \tag{14.2}$$

Linear regression of a [time, potency] data set to Eq. 14.2 will provide estimates for the slope and y-intercept, representing, respectively, the estimated rate constant for the loss of potency, k, and the natural log of the value of the potency at $t = 0$, $\ln P(0)$. As an example, the data from Fig. 14.2 (see Appendix) may be analyzed for a particular lot, for example, Lot 1. The least-squares analysis provides the best estimates of the parameters of the linear model and the uncertainties associated with those parameters (see Fig. 14.3). The regression line crosses the lower limit for potency at *ca.* 27 months, a value that might reasonably be regarded as a shelf life. The ICH Guidance on stability (Q1E) recommends, however, that the uncertainty in the regression analysis needs also to be considered. Thus, given a decrease in potency over time, the shelf life may be set as the point in time where the one-sided 95 % confidence limit on the regression line intersects the prescribed lower limit for potency—approximately 22 months for Lot 1.[3] (The dashed line in Fig. 14.3 presents

[3] ICH Q1E notes that, "for an attribute known to decrease with time, the lower one-sided 95 % confidence limit should be compared to the acceptance criterion. For an attribute known to increase with time, the upper one-sided 95 % confidence limit should be compared to the acceptance



	Lot # 1
Best-fit values	
Slope	-0.02364 ± 0.003705
Y-intercept when X=0.0	8.637 ± 0.07165
X-intercept when Y=8.0	26.97
1/slope	-42.31
95% Confidence Intervals	
Slope	-0.03240 to -0.01487
X-intercept when Y=8.0	21.96 to 35.69
Goodness of Fit	
r^2	0.8532
Sy.x	0.1310
Is slope significantly non-zero?	
F	40.70
DFn, DFd	1.000, 7.000
P value	0.0004
Deviation from zero?	Significant

Fig. 14.3 Linear least-squares fit of time and potency data set for *Lot 1*. The *regression line* is the *solid line* and the 90 % confidence interval about that *regression line* is represented by the *dashed lines*. The least-squares values from the regression analysis are presented in the accompanying Table. In the accompanying Table, "Sy.x" represents the standard deviation of the residuals

Table 14.1 Calculated slope and intercept, with associated standard deviations, for Lots 1, 2, and 3, as well as combined Lots 1, 2, and 3

Lot number	Slope	Standard deviation of the slope	Intercept	Standard deviation of the intercept
1	−0.0236	±0.0037	8.637	±0.0716
2	−0.0194	±0.0027	8.601	±0.0521
3	−0.0207	±0.0034	8.629	±0.0663
1, 2, 3, combined	−0.2125	±0.0018	8.622	±0.0487

the two-sided 90 % confidence limits about the regression line; the lower branch of the two-sided 90 % confidence limit is equivalent to the lower one-sided 95 % confidence limit. A similar analysis can be carried out for Lots 2 and 3 and, as can be noted in Table 14.1, slightly different estimates for the rate in potency loss are observed, with slightly decreased rates of potency loss seen for Lots 2 and 3. Under suitable conditions, as defined in ICH Stability Guidance (ICH Q1E), the data from various vaccine Lots may be combined to obtain a better estimate of the parameters of the kinetic model.[4] This was done for the three Lots (which meet the ICH criteria for

(Footnote 3 continued)
criterion. For an attribute that can either increase or decrease, or whose direction of change is not known, two-sided 95 % confidence limits should be calculated and compared to the upper and lower acceptance criteria". In the examples of the time dependence of potency presented in this chapter, a loss in potency with time is considered.

[4] Analysis of covariance (ANCOVA) can be employed, where time is considered the covariate, to test the differences in slopes and intercepts of the regression lines among batches. Each of these

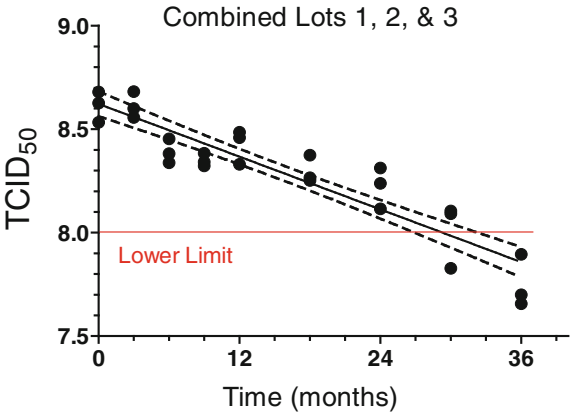


Fig. 14.4 Least-squares regression analysis of *Lots 1*, *2*, and *3* combined. The *dashed lines* represent the two-sided 90 % confidence limits of the *regression line*

combining of the data sets) of this example and the results are displayed in Fig. 14.4 (see also Table 14.1). Through the combination of the three Lots, it can be seen that the confidence limits about the regression parameter estimates have decreased. An additional comment about the results of these data analyses may be made. For any given time point over the vaccine shelf life, the potency estimate that is provided by the regression line may be regarded as a better estimate of the potency at that time than the measured value (for example, see Fig. 14.3) or the average of the measured values if replicate determinations are made (for example, see Fig. 14.4). This consideration is of importance when considering potential "out-of-specification" values at any given time point, and especially at time points approaching the end of shelf life for those vaccines that exhibit a loss of potency over time.

As noted, the regression analysis is based on a mathematical model. Various results from the regression analysis may be used to assess the adequacy of the model; for example, an analysis of the residuals (the residuals should be randomly distributed about the regression line and their standard deviation should be approximately equal to the SD of the potency assay).

The value of the coefficient of determination (the r^2 value) or the correlation coefficient (r) is often taken as a measure of the adequacy of the linear model. However, as is noted in the following Sect. 14.2.2.1, there are concerns associated with the sole use of this evaluation metric.

As shown in Fig. 14.4, the regression line intersects the lower assigned potency limit at nearly 30 months, while the lower one-sided 95 % confidence limit intersects the lower limit at approximately 27 months. These are slightly different values than were obtained from the analysis of Lot 1 (or of Lots 2 and 3).

(Footnote 4 continued)
tests should be conducted using a significance level of 0.25 to compensate for the expected low power of the design due to the relatively limited sample size in a typical formal stability study.

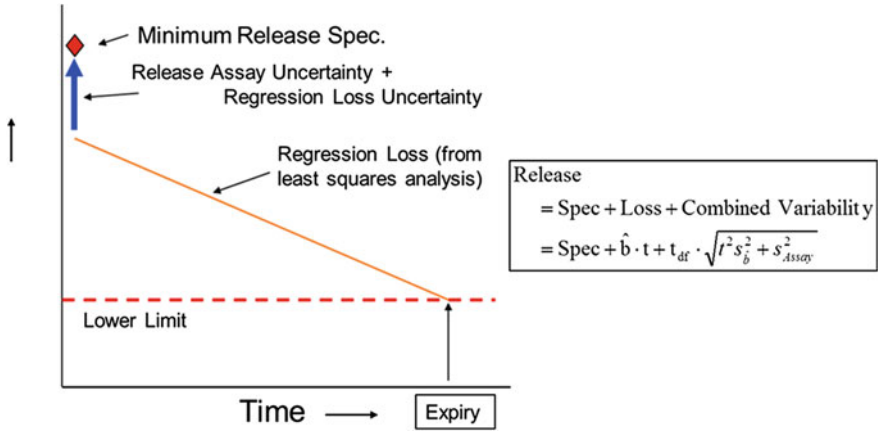


Fig. 14.5 Calculation of a minimum release specification as presented in the WHO Guidelines of stability for vaccines. The release specification is determined by the estimated rate of loss of potency over time as well as the uncertainties in the estimation of that rate of potency loss and the release assay

14.2.2 Setting a Shelf Life and Associated Lot-Release Specification

In the introduction of this chapter, we noted that, although commonly used, several drawbacks attend the use of a "compliance model" to setting a release specification and shelf life. We now present an alternative paradigm, the "Expiry Model", which has also been described in the aforementioned WHO Guideline on Vaccine Stability. The various elements of the Expiry Model are illustrated in Fig. 14.5. Basically, with a target shelf life as a goal, a minimum release value is calculated by accounting for the loss of potency over the period of the proposed shelf life and the combined uncertainties in the rate of potency loss and the determination of the potency at release. Knowing the rate at which potency is lost (from the least-squares regression analysis), a potency value at $t = 0$ is determined (the y-intercept at $t = 0$); uncertainties in (i) the rate of potency loss and (ii) the release potency assay are then added in. The combined uncertainty is given as a statistical multiplier (associated with 95 % confidence[5]) times the square root of the sum of the variances of the individual determinations for the rate of potency loss and the release potency assay. Thus, we have:

$$\text{Release Potency} = \text{Lower Potency Limit} + \hat{b}_1 \times t + t_{\alpha,df} \sqrt{(t \times s_b)^2 + (s_a)^2}$$

[5] Although other confidence limits might be chosen, 95 % is a generally agreed-to default value.

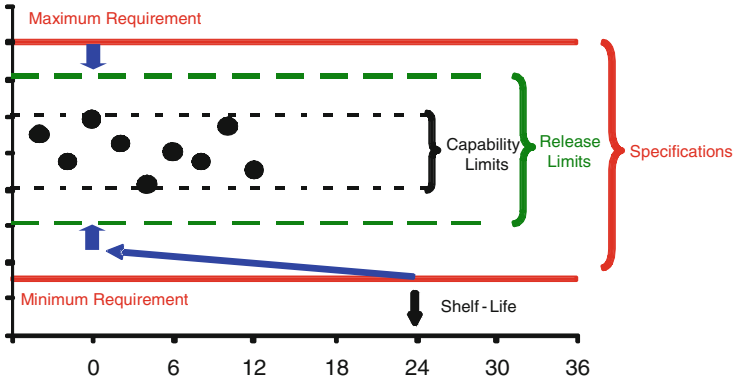


Fig. 14.6 Ideal relationship between the minimum release value and manufacturing window

where

$\hat{b}_1$ is the least-squares slope of the regression line
t is the shelf life
$t_{\alpha,df}$ is the critical t-value for the desired certainty with the degrees of freedom associated with the regression
s_b is the standard deviation of the regression line slope
s_a is the standard deviation of the release assay potency.

Similarly, an upper release limit can be calculated to assure (with 95 % confidence) that the true potency does not exceed an upper bound.

Ideally, the thus-calculated release potency value will embrace the range of the manufacturing capability. If, however, the lower release limit is not less than the lower value of the manufacturing capability, it will be necessary to either decrease the shelf life and recalculate a minimum release limit or, alternatively, reject those Lots where the manufactured value falls below the needed release value. This relationship between manufacturing capability and release value is illustrated in Fig. 14.6.

14.2.2.1 A Statistical Interlude

For purposes of establishing an estimate for the potency loss over time, as shown in Figs. 14.3 and 14.4, the mathematical expression describing the loss of potency, Eq. 14.1, was first transformed to a linear form, Eq. 14.2; the transformed data were then analyzed to provide estimates for k and $P(0)$. While the transformation provides an equation which can be fit using simple linear regression, this logarithmic transformation also generates data that are commonly more suitable for statistical modeling. An assumption of least-squares linear regression is that the variance is uniform across levels of response and that the error values (the differences between

the measured and fit values) are normally distributed. Potency measurements are frequently log-normally distributed. A log transformation of such measurements will therefore have attributes which satisfy the assumptions of the modeling.

In Figs. 14.3 and 14.4, in addition to providing the least-squares regression line, the confidence interval (CI) about that regression line was provided (the dashed lines in the Figures). The confidence interval represents the uncertainty in the estimation of the kinetics model. In this regard, the point in time where the lower confidence bound intersects the minimum potency requirement is the maximum time that we are assured (assured with probability ≥0.95) that the vaccine remains above the minimum potency (Fig. 14.5).

In selecting a set of time points for the determination of the rate of potency loss, time points presented by the ICH Guidance on stability, were used (these are generally 0, 3, 6, 9, 12, 18, 24, 36, 48 months). Although generally regarded by regulators and manufacturers as an acceptable set of time intervals, from the point of view of minimizing the error in the estimated shelf life, they are not always ideal. The equation for the lower confidence bound (at time t) is as follows:

$$\mathrm{LCB}(t) = \hat{y}(t) - t_{\alpha,\mathrm{df}} \cdot s \cdot \sqrt{\frac{1}{n} + \frac{(t-\bar{t})^2}{\sum (t_i - \bar{t})^2}}$$

where

$\hat{y}(t)$ is the estimated response from the linear regression equation,
$t_{\alpha,df}$ is a critical value from the *t*-distribution,
s is the regression means square error (an estimate of assay variability), and
$\bar{t}$ is the average time

The limiting factor in this equation is: $\frac{(t-\bar{t})^2}{\sum (t_i-\bar{t})^2}$. This is reduced by either decreasing the numerator or increasing the denominator. The numerator is minimized by concentrating time points near the expected shelf life, *t*. It will be shown later that the denominator is maximized by concentrating time points at the beginning and end of the shelf life period.

The point of showing that alternative designs may provide better estimates of the shelf life than ICH is not to argue against using ICH intervals. These are de facto regulatory intervals. Rather the point is to illustrate that better estimates can be achieved in stability studies through reduction of uncertainty (variability), which can be managed through stability study design. For example, this may be achieved by addition of extra data points near the expected time of expiry or past the expected time of expiry.

We have noted that the simplest plausible kinetic model for loss of potency would be a first-order kinetic model. However, the loss of potency for any particular vaccine may not follow first-order kinetics and more complicated kinetic models may be necessary. The first question we should address is whether a first-order kinetic model is *adequate* to describe the data and then whether a first-order kinetic

model is able to predict the potency at a later time point. To the first part of this question, statistical tests may be applied to the model, namely, we can ask whether the log-transformed data are linear. A common statistic r^2 (r-square) is not ideal for detecting nonlinearity. This is because r-square is impacted by a number of factors: (1) the steepness of the slope; (2) the variability about the regression (s); and (3) the variability (range) in the time points. The variability about the regression (s) is closest to capturing the aspect of graphical linearity, and may therefore be used to monitor linearity. Alternatively one may use residual plots or fit a model with a quadratic (curvature) term in the model. While a test of significance of the quadratic term would indicate statistically significant curvature, an equivalence approach using a range on the quadratic coefficient (equivalence margin) is better.

14.2.2.2 A Chemical Kinetics Interlude

At times, it may appear that it is not necessary to log-transform the data; that is, that the data are adequately fit by a simple linear model. Chemically, this would correspond to the vaccine losing potency via a zero-order kinetic model, wherein $P(t) = P(0) - kt$. Although zero-order reactions are rare, and generally derive from surface catalysis, the appearance of zero-order like kinetic behavior is not surprising as it mimics the initial portion of an exponential process, i.e., first-order kinetics. The similarity between a zero-order reaction and the initial portion of a first-order reaction is readily seen by expanding the exponential (see Eq. 14.2) in a Taylor series and noting the close correspondence of time values to a zero-order model when

$$e^{-kt} = 1 - \frac{kt}{1!} + \frac{(kt)^2}{2!} - \frac{(kt)^3}{3!} + \cdots$$

values of kt are small; see Table 14.2.

Although a zero-order model may be seen to adequately fit the available data, we note that such a model would be highly unusual from a chemical kinetics viewpoint, but, more importantly, that extrapolating zero-order kinetics to longer times, for which data are not available, would inappropriately lead to a markedly reduced estimate for the shelf life. This example also serves as a caution in overly relying on extrapolated data, even when the model appears to provide a reasonably good fit to the existing data.

Table 14.2 Values of $A_0 \exp(-kt)$ and $A_0[1-kt]$ as a function of kt

kt	$A_0 \exp(-kt)$	$A_0[1-kt]$
0.05	95.1	95.0
0.10	90.5	90.0
0.15	86.1	85.0
0.20	81.9	80.0
0.25	77.9	75.0

14.3 The Temperature Dependence of Reaction Rates and the Arrhenius Equation

The rates of chemical reactions are, in general, dependent on temperature, increasing with increasing temperature. It is a relatively well-accepted generalization, about which we will soon have more to say, that the rate of a reaction approximately doubles with each 10° increase in temperature. As changes in vaccine potency derive ultimately from chemical transformations, potency changes are also temperature dependent. This temperature dependence is generally described in terms of the Arrhenius[6] equation, shown below:

$$k = Ae^{-E_a/RT} \tag{14.3}$$

where

k is the reaction rate constant
R is the universal gas constant
T is the temperature in degrees Kelvin
E_a is the activation energy for the reaction
A is the pre-exponential factor (the units of A are those of the rate constant, k; for a first-order reaction, the units are time^{-1})

The values of E_a and A are considered constant for a given reaction, a reasonable approximation given the relatively limited temperature range that is generally investigated in kinetic studies of vaccines. Therefore, if E_a and A are known, the reaction rate at any temperature may be calculated. The values of E_a and A may be determined measuring the reaction rate at two different temperatures, generating two equations for the two unknowns. In practice, the reaction rate at a number of temperatures is determined and the [rate, temperature] data set are then analyzed by a least-squares fitting procedure to a linearized form of the Arrhenius equation, gotten by taking the natural log of both sides of Eq. 14.3; thus

$$\ln(k) = \ln(A) - \frac{E_a}{R} \cdot \frac{1}{T} \tag{14.4}$$

From a least-squares fit of $\ln(k)$ versus $1/T$, one obtains a slope, Ea/R, and intercept, $\ln(A)$; see Fig. 14.7. With the values of E_a and A in hand, as noted above, the rate constant at any other temperature may be calculated, either within or outside of the investigated temperature range; it is important to note, however, that the further outside of the investigated temperature range, the greater the uncertainty in the predicted reaction rate, as indicated by the 95 % confidence limits about the regression line shown in Fig. 14.7. It should be noted that the accuracy of the

[6] The relationship between reaction rates and temperature was developed by the Swedish physical chemist, Svante August Arrhenius (1859–1927).

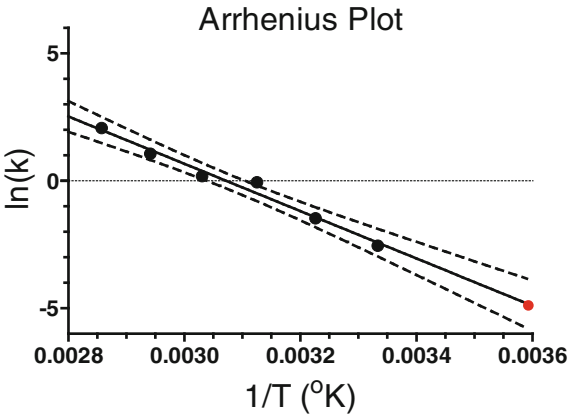


Fig. 14.7 A plot of ln(k) versus 1/T for a hypothetical vaccine displaying Arrhenius-type behavior. From such a plot, values of E_{act} and A may be obtained. An extrapolated rate, at a lower temperature, is shown in *red*

simple least-squares fit presented in Fig. 14.7 is premised upon determining the reaction rates at each temperature with the same degree of accuracy; since reaction rates decrease with decreasing temperature, increasing amounts of time are needed to monitor a fixed level of change (and hence constant error in the rates) as the temperature decreases. Because of time constraints, this is seldom done and more sophisticated statistical approaches should be considered, namely, attaching appropriate weighting factors to the various the data points.

As noted above, the statement is generally made that reaction rates increase approximately twofold for each 10° increase in temperature. Although a useful rough approximation, the actual increase in rate depends on the value of the activation energy and on the temperature range that is investigated. For two specific temperatures, T_1 and T_2, we may write the Arrhenius equation as:

$$\ln(k_1) = \ln(A) - \frac{E_a}{RT_1} \tag{14.5a}$$

and

$$\ln(k_2) = \ln(A) - \frac{E_a}{RT_2} \tag{14.5b}$$

Subtracting Eqs. 14.5a from 14.5b, we arrive at the expression:

$$\frac{k_2}{k_1} = \exp\left\{\frac{E_a(T_2 - T_1)}{RT_2T_1}\right\} \tag{14.6}$$

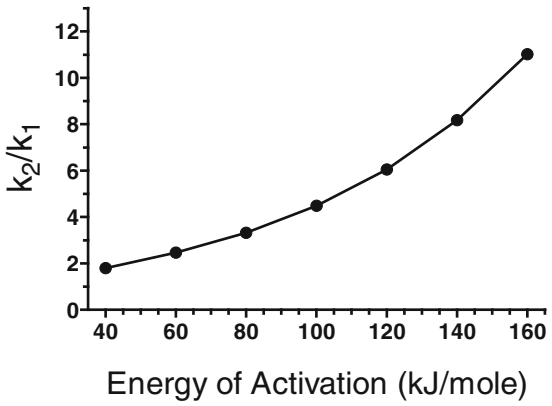


Fig. 14.8 The *n*-fold increase in reaction rate due to a 10° increase in temperature, from 5 to 15 °C, as a function of the energy of activation

The *n*-fold increases in rate (the value of k_2/k_1) as a function of selected values for the activation energy for an increase in temperature from 5 to 15 °C are displayed in Fig. 14.8. We also note that as the average temperature for the 10° interval increases (for example, from 100 to 110 °C vs. 5 to 15 °C), the *n*-fold change in rate will decrease.

As noted, the value of having established the Arrhenius parameters is that the reaction rate may then be calculated at any temperature. Thus, one may determine the Arrhenius parameters at high temperatures, where reactions occur rapidly, and then calculate the expected reaction rate at lower temperatures, where reactions occur slowly. Thus, one may determine in several months, what might otherwise require several years. This is illustrated in Table 14.3, where, for a first-order reaction, and a representative value of E_{act} and A, the change in rate is provided as a function of temperature; the change in the reaction half-life[7] is also provided in the Table.

The question may be raised why, given this potentially enormous saving in time, that accelerated kinetics are not used in establishing a product shelf life. There are two major reasons why this is not done. The first is a practical one. Due to experimental errors in determining the reaction rates at various measured temperatures, there is an associated uncertainty in the determined Arrhenius parameters and, accordingly, uncertainties in the extrapolated rates; the greater the extent of the extrapolation, the greater the uncertainty. In general, a sufficiently accurate shelf life can simply not be gotten by any practical use of the Arrhenius equation. There is also a theoretical component, in that Arrhenius behavior may only be approximately followed; that is, the Arrhenius parameters may themselves be temperature dependent. Although the Arrhenius equation may not be used for setting a shelf life, it does have a number of valuable uses. First, it is of considerable values in developing a vaccine formulation, where the primary concern is an increased shelf

[7] The half-life for a reaction is the time taken for one-half of a reactant to be consumed; for a first-order reaction, the half-life, $\tau_{½}$, is equal to ln (2)/k.

Table 14.3 Rate constants and associated half-lives for a reaction with an E_{act} of 90 kJ/mole and pre-exponential factor, A, of 1×10^9 s^{-1}

Temperature (°C)	Rate constant, k	Half-life, $\tau_{½}$ (months)
5	3.2×10^{-2}/month	21.6
15	12.3×10^{-2}/month	5.6
25	43.4×10^{-2}/month	1.6
35	131.4×10^{-2}/month	0.5

life. The trends that are observed at higher temperature will, in nearly all cases, persist at lower temperatures. Thus, a formulation that provides greater stability at 35 °C will provide a greater stability at 5 °C. A comparison between two formulations may be carried out in several months at 35 °C, whereas the comparison might require several years at 5 °C. Second, an accelerated stability study is of considerable use in establishing product comparability following a manufacturing change. If the vaccines are comparable at a higher temperature, for example 35 °C, they are very likely to be comparable at 5 °C. Again, observing a change and showing that the change is comparable in the two vaccines, is more readily accomplished at the higher temperature(s). Finally, from values of the Arrhenius parameters, one may estimate the extent of potency losses that may occur during excursions from given storage conditions.

14.4 Stability Studies and the Product Life Cycle

The goals of stability studies vary during the product life cycle. Prelicensure, at the earlier stages of product development, the goals of stability studies are related to knowing stability over the course of clinical trials (and thus knowing the potency of vaccine that clinical trial subjects will receive and have received) and developing a vaccine formulation that maximizes product stability. At the time of licensure, the goals of stability studies are related to establishing a shelf life and release specifications as well as demonstrating manufacturing consistency. Postlicensure, the goals of stability studies are to demonstrate manufacturing consistency as evidenced by similar potency profiles over time (i.e., the annual stability studies) and to support the comparability of the vaccine following manufacturing process changes.

Prelicensure and licensure. As noted above, at the prelicensure stage of development, the goals of stability studies are to describe the vaccine stability over the course of clinical trials, to develop a formulation that maximizes the stability of all active components of the vaccine, and to demonstrate manufacturing consistency through a consistent stability profile. Finally, a shelf life, under defined storage conditions, must be established at the time of licensure.

As stated, vaccines are formulated to maximize stability. For this purpose, namely, developing a formulation that maximizes vaccine stability, it is useful to carry out the stability studies at temperatures that are higher than those that are intended for storage. At the higher temperatures, changes in vaccine potency are

detected more rapidly and, generally, more precisely. The trends in stability at the higher temperatures will, with rare exception, remain at the lower temperatures; that is, the more stable formulation at the higher temperature will be the more stable formulation at the lower temperature (as illustrated in Fig. 14.9). Indeed, based on the previous discussion of *n*-fold changes in rates with temperature, the ratio of rates would be expected to become slightly more disparate at lower temperatures. Although it would be sufficient to study the various formulations at a single higher temperature, it is, nonetheless, useful to study a range of temperatures, both to demonstrate that Arrhenius behavior is followed over the studied temperature range (for example, 15–45 °C) as well as to actually determine the Arrhenius parameters, E_{act} and A, so as to be better able to extrapolate kinetic behavior to the lower temperatures (and, thus, an estimate for the expected shelf life) and to be able to estimate stability at intermediate temperatures (and thus be able to determine the consequences of unexpected temperature excursions).

During the final phases of clinical development, it is necessary to demonstrate manufacturing consistency, for which stability studies play an important role. In this regard, it is necessary to select the best metric for demonstrating consistency. A relevant metric of consistency might be a comparison of the slopes of the regression lines from the stability studies of the various vaccine Lots (generally three Lots are evaluated for manufacturing consistency). While this seems a reasonable choice of metric, a question then arises as to the basis for comparing the thus-obtained slopes. The slopes might be compared for poolability as outlined in the ICH Guidance (Q1E). Alternatively, the slopes might be evaluated for equivalence within a predefined margin. A fuller discussion of these two approaches is provided in the next section on postlicensure stability studies.

At the time of licensure, a shelf life for the vaccine, under defined storage conditions, needs to have been established. This shelf life must be established from data gathered under the intended storage conditions and over the period of the intended shelf life. Some extrapolation of the data in establishing the shelf life may be warranted; however, further data to justify the extrapolation would then be needed. In practice, to reduce the error in estimating the shelf life, having additional

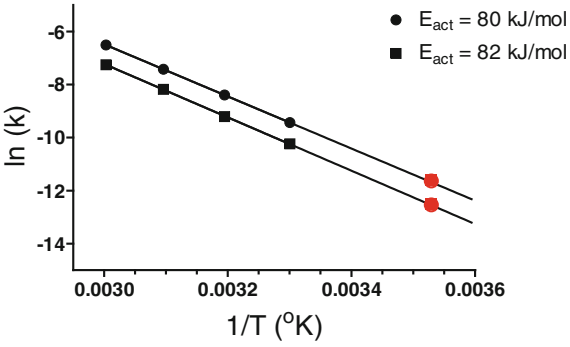


Fig. 14.9 Arrhenius plots of two hypothetical vaccine formulations having slightly different energies of activation for a process leading to a loss of potency

data points near the intended expiry period (additional to those indicated by the ICH Guidance) as well as having data from time points in excess of the intended shelf life are useful.

Postlicensure. Postlicensure, annual stability studies are required. Each year randomly selected vaccine Lots are required to undergo stability testing over the product's shelf life. The goal of these studies is primarily to demonstrate that the stability profile of the postlicensure produced vaccine is comparable to that of vaccine produced at the time of licensure and to provide ongoing support for the shelf life that was established at the time of licensure (the precise goals of postlicensure stability studies have not been delineated in either regulation or guidance).

With this goal in mind—demonstrating comparability to previously manufactured Lots—the question may be raised as to what might constitute the best metric for defining comparability. One commonly employed approach is to simply demonstrate that the vaccine maintains its potency at all measured time points (generally, those stated in the ICH Guidance), falling between the established upper and lower limits. This is akin to the "compliance model" that was previously mentioned. While this may help to confirm the appropriateness of the designated shelf life (assuming that the potency for all time points remain within the upper and lower bounds), several problems attend this approach. The first is that an actual change in stability, albeit one that is consistent with the assigned shelf life, may be overlooked. From a cGMP perspective, this is undesirable. If there is a change in the stability profile, it should be known and further studied to determine its root cause and potential effect on other vaccine attributes.[8] The second problem relates to an observed out-of-specification value, a situation that becomes increasingly more probable as the expiry period is approached. The question may be posed whether the product actually is out of specification because of a change in the product stability profile, or, alternatively, appears to be out of specification because of the variability in the potency assay. The potency at that time point or a subsequent time point may be remeasured. If the value for the subsequent measurement is within specifications, the problem of deciding which measurement is "correct" remains. The results of additional measurements may help to assure certainty in a decision; however, such additional testing may require a significant amount of time, especially if the potency assay is animal based. The point to be made, however, is that there is additional stability information that is contained within the previous potency determinations and these should be brought to bear on this latter problem.

A more meaningful metric of comparability may be to compare the rates of potency loss of prelicensure Lots of vaccine (and that were used in efficacy studies) with those being currently marketed. In such an exercise, the slopes from the respective regression analyses would be compared. This comparison might be made in different manners. One might, for example, employ methods and acceptability criteria to determine if the data were "poolable", i.e., the slopes were parallel, as in

[8] Although potency tests are designed to indicate the expected effects in the intended recipient population, they are, in reality, often imperfect predictors.

the ICH Q1E Guidance. Alternatively, the data might be the subject of an equivalence test. We would like to comment on these two methods. The above-mentioned test for poolability, while often used, suffers from a significant drawback. On the one hand, for regression analyses having scant and variable data, one is not able to conclude that the slopes are different and hence the data are regarded as poolable; on the other hand, for regression analyses with abundant and precise data, minor differences, which may not be practically significant (as opposed to statistically significant) from a consideration of vaccine use, will be detected and the data will be considered noncomparable. These possibilities are illustrated in Fig. 14.10.

Thus, there is seen to be a risk of masking real differences due to data variability or inadequate study design or the risk of highlighting an insignificant difference when the assay is precise or there is an adequate study design. Such an approach appears counterintuitive in that there is a reward for excess variability and a penalty for good precision. An alternative approach would be to evaluate the equivalence of the slopes within predefined limits, as illustrated in Fig. 14.11.

Postlicensure, various manufacturing changes are made, for which it is necessary to demonstrate product comparability. Stability studies form an integral part of that comparability study. The basic principles that have been given above for evaluating annual stability studies will equally apply to comparability studies following manufacturing changes. Of course, some changes are made to enhance product stability and it would then be necessary to demonstrate a superiority.

Returning to the theme of demonstrating comparability, either in the annual stability studies or following manufacturing changes, and accepting use of the slope as a metric for comparability, the question may be posed as to whether that comparability should be demonstrated at the intended storage temperature or at an elevated temperature, or both. In general, stability studies at elevated temperatures form part of the comparability assessment following manufacturing changes; data under the intended storage conditions are also utilized and do form the primary basis for the assigned shelf life. In contrast, the annual stability studies are carried out under the licensed storage conditions, generally 5 ± 3 °C. For a fixed time increment, there will be a greater change in vaccine potency that is observed at a

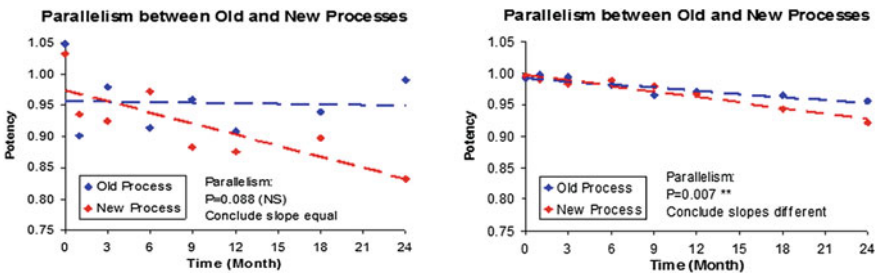


Fig. 14.10 Hypothetical time, potency data sets wherein a significant difference in rates of potency loss may be masked by limited, imprecise data (*left hand panel*) or minor difference in potency loss is considered statistically significant because of precise data (*right hand panel*)

Fig. 14.11 A comparison of the slopes obtained from linear least-squares regression analyses of two vaccine Lots. Having selected a Δ-value that is considered as an allowable variation in the rate of potency loss relative to a reference value (indicated by the *shaded rectangle*), the confidence intervals of the test samples may be compared. The *blue line* depicts a comparable value in that the depicted confidence interval about the point estimate for newly measured slope is within the agreed-to range; the *red line* depicts a noncomparable value in that the confidence extends outside this range

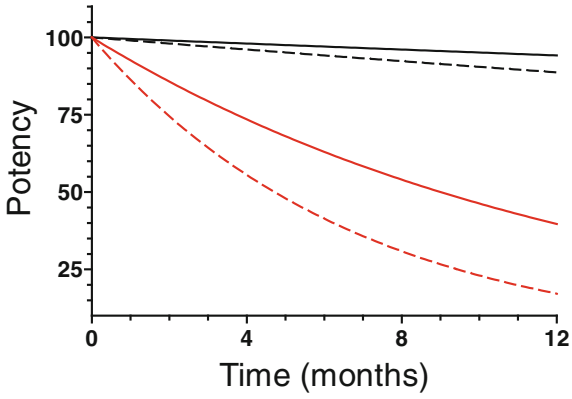


Fig. 14.12 Depiction of a twofold change in the rate of potency loss as might occur at a low temperature (*black lines*) and as that twofold rate change might appear at an elevated temperature, corresponding to a 20° increase in storage temperature (*red lines*)

higher temperature than at a lower temperature; for example, for a vaccine studied at 5 and 25 °C, we might expect an approximately 10 to 20-fold decrease in potency at 25 °C relative to that observed at 5 °C. Given that the error in measuring potency is the same for all samples, e.g., whether there has been a 5 % loss in potency or a 50 % loss in potency, then the ability to detect a change in the rate of potency loss is greater for the sample that has undergone the greater change. An argument might therefore be advanced that annual stability studies might best be carried out at elevated temperatures, provided the goal of such studies is to monitor the consistency of manufacture (as evidenced by the consistency of the stability profile). This is illustrated in Fig. 14.12. Consider a vaccine that had a modest loss in potency (approximately 6 %/year; $k = 0.005$ month^{-1}) and that, due to an unsuspected manufacturing variation, the rate doubled.[9] Given a modest uncertainty in potency

[9] In the above example, activation energies, E_a, of 94.28 and 92.67 kJ/mole (with a pre-exponential factor of 10^{-9}) were chosen to mimic the kinetics at 5 °C and to subsequently calculate the rates at 25 °C.

determinations, it would be difficult to detect a twofold change within 1 year at 5 °C, as is illustrated by the solid and dashed black lines in Fig. 14.12. For this hypothetical vaccine having the Arrhenius parameters presented in Footnote 6, a 20° increase in temperature would lead to an approximately 15-fold increase in rate as depicted by the solid and dashed red lines in Fig. 14.12. The twofold change in rate at 5 °C would be more likely to be detected at the increased temperature within the 12-month period and, possibly, sooner. In summary, a reasonable case may be advanced for conducting annual stability studies at elevated temperatures, either in lieu of studies at the storage temperature or in addition to them.

14.5 Concluding Remarks

The principle goal of all stability studies is to ensure vaccine quality throughout the vaccine's shelf life. This entails having established an acceptable lower limit for potency and knowing the rate at which the vaccine loses potency, and, finally, accounting for the uncertainties in the determination of potency at release and the rate of potency loss. Done properly, such stability studies reduce the risk to the vaccine recipient of receiving a subpotent vaccine while reducing the risk to the manufacturer of rejecting suitably potent vaccines.

Stability studies begin with establishing an appropriate mathematic model for the data; in this chapter, we have exemplified various principles using a first-order kinetic model, the simplest feasible model for loss of vaccine potency; we have noted that more complex models may be needed—for example, biphasic kinetics or second order kinetics (as might be encountered in protein oxidation). Various statistical tests to determine the adequacy of the model are then employed—for example, testing for linearity of the log-transformed data or evaluating that the residuals are normally distributed. Given the adequacy of the kinetic model, the uncertainties associated with the key parameters can be determined; confidence limits on the slope of the regression line can be determined and, together with the known variance of the release assay, can be used to establish a lower release limit and shelf life for the vaccine.

Although the shelf life of the vaccine needs to be established at the intended storage temperature, the use of accelerated degradation studies (at higher temperatures than intended for storage) were seen to be useful in developing a vaccine formulation to optimize stability and in situations where it is necessary to establish the consistency or comparability of the vaccine's stability profile. From a study of the vaccine's degradation rate as a function of temperature, the Arrhenius parameters for the loss of potency can be determined and used to estimate vaccine stability at other temperatures (although it is not used in establishing the shelf life).

The importance of statistical considerations have been emphasized in this chapter. Such considerations are necessary for understanding the risk that is associated with interpreting stability data and establishing a lower release limit. A basic principle in evaluating stability that has been emphasized in this chapter is that a set

of measurements are collected over time to provide estimates of stability parameters (rates of potency loss and the associated uncertainty in those rates) and not to assess compliance of individual data points with specifications. Measuring uncertainty in connection with stability estimates places an emphasis on the design of experiments together with the goals of the study, for the end purpose of delivering high quality vaccines.

14.6 Appendix

See Table 14.4.

Table 14.4 Time and potency data for Lots 1, 2, and 3 as shown in Fig. 14.2, prior to logarithmic transformation

Time (months)	$TCID_{50}$ for Lot 1	$TCID_{50}$ for Lot 2	$TCID_{50}$ for Lot 3
0	5082	5882	5575
3	5201	5893	5431
6	4693	4177	4367
9	4376	4193	4118
12	4844	4148	4718
18	3885	3836	4335
24	4073	3346	3783
30	2507	3309	3268
36	2115	2684	2208

Chapter 15
Vaccine Reference Standards

Brian K. Nunnally

15.1 Philosophy

The characterization and use of vaccine reference materials are the most important responsibilities of the vaccine analytical laboratory. This could be considered an odd statement given the importance placed on potency and content. Minimizing variability is important in all vaccine analytical analysis; this is especially true of reference standard characterization. If variability is allowed to run rampant through the characterization of a reference standard, trouble will ensue. Since nearly all of the numbers generated by the laboratory depend on the standard used, the consequences of poorly characterized and improperly used reference materials are dire. The customers (i.e., the production unit, the patients) depend on the laboratory to understand and minimize variability associated with reference materials. Proper characterization does not happen by accident; it requires close oversight and careful analytical scrutiny. Once the standard is characterized, the oversight does not end. It is needed while the material is being used, on an ongoing basis. This is good science. Too many issues, as discussed in the case studies, can be traced back to standards which are not closely monitored.

15.1.1 Factors to Consider When Selecting a Reference Material

There are several factors to consider when selecting a vaccine reference material (Note: the terms reference material, reference standard, and standard will be used interchangeably throughout this chapter. There is no intended difference in the

B.K. Nunnally (✉)
1201 Fairfax Woods Drive, Apex, NC 27502, USA
e-mail: usc4duke@yahoo.com

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6_15

meaning of these terms.) First, it is critical for the standard to be stable, at least for a reasonable time. Standards which are unstable can be used, but the constant changing of standards will inject additional variability into the assay. When this is unavoidable, sufficient work needs to be performed to ensure any shifts in the data are expected and communicated. A standard should be pure. It is possible to use an impure standard, but this adds an additional characterization burden and increases the likelihood of getting a matrix effect. The standard should have sufficient supply. Determining the amount of material needed for a standard is an inexact science. Some factors to consider are discussed in the section below. Finally, the standard needs to be representative of material being analyzed. This seems an obvious point, but it is a point that is often neglected. When the standard is not representative, it creates questions about the ability of the standard to provide proper quantitation of the samples analyzed. These can be avoided by ensuring representative materials are selected.

One additional consideration is related to wishes of the regulatory agencies. The European Medicines Agency (EMA) and Food and Drug Administration (FDA) prefer the use of standards which have been in the clinic or can be tied directly to the pivotal clinical lot(s).

15.1.2 How Much Do I Need?

The amount of material needed for a standard depends on many factors including the usage rate (e.g., how much do I use), the amount of standard in each vial (e.g., how much do I need each time I use it), the stability of standard (e.g., how long will it last), and the amount of available material (e.g., how much can I get). Multiplying the number of assays per month (usage), the amount of standard in each vial, and the months of stability together provide an overall amount of standard needed to be determined. The next step is to add an overage amount to account for the uncertainty in the factor estimates. The overage should be anywhere from 25 to 100 %, depending of how much uncertainty went into the calculations. This is the amount of material to request, assuming this amount of material is available. In many vaccine companies, the material must be bought (internal accounting). Good standards (and controls) are so valuable that this cost should be paid, as it is crucial to the laboratory's aim to produce quality data.

15.1.3 Research and Development Versus Commercial

Vaccine research and development groups often have different needs than commercial vaccine laboratories when it comes to standards. The process for characterizing standards in development is different from marketed products. In many cases, the assays used to characterize the materials are still in development (or do

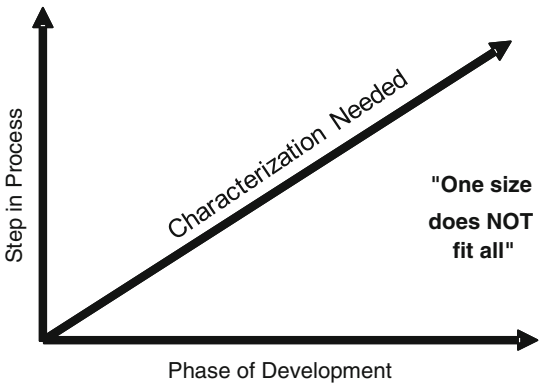


Fig. 15.1 Characterization required by development phase

not exist), the material availabilities are limited (or non-existent), and the understanding of the critical attributes is still years away. Vaccine research and development groups have to be more flexible in the materials used for standards. Sources for development reference standards include lab-scale lots, demonstration batches, and development batches (e.g., clinical batches, manufacturing runs, etc.). Ultimately, the most important caveat is that the material must be representative of the current process. This may mean that materials need to be changed as the process continues to develop and change.

The closer the process gets to commercialization, the more characterization required. This is illustrated diagrammatically in Fig. 15.1. The amount of characterization needed for a vaccine research and development fermentation standard is quite different from a commercialized drug product standard. One kind of characterization does not fit all situations.

15.1.4 Monovalent or Polyvalent

For polyvalent vaccines, will the standard be a monovalent or a multivalent? This is an important consideration. Either type of standard is possible depending on the application. It often makes sense to make both types to take advantage of when each is needed.

15.2 Types of Standards

There are several different types of vaccine standards. Not all types of standards are applicable to all industries, but there are some common elements which cross industries.

15.2.1 Compendial/Regulatory

Compendial (or Regulatory) standards are produced by governmental or widely accepted standard setting bodies (e.g., National Institute of Standards and Technology, United States Pharmacopeia). Groups such as the American Type Culture Collection (ATCC) fit into the category as well. There are several advantages to this type of standard. They are considered to be reliable and their use as a standard is not questioned by regulatory or certification bodies. They are always well characterized and this characterization work does not have to be done by the laboratory using them. In nearly all cases, there is sufficient supply. Unfortunately, these types of standards are not available for all vaccines or intermediates and they can be expensive. In many cases, it is better to use them as a primary standard and create secondary standards from material produced in-house.

15.2.2 Primary

Primary standards are reference materials that are accurate enough to not require calibration or be considered subordinate to other reference materials. Primary standards are often created when compendial or regulatory standards are not available. The source material is made in-house and should be well characterized. By spending the effort on a primary standard, this allows for significantly reduced characterization in subsequent (secondary) lots. This is also a disadvantage since significant resources are needed to characterize these materials. In addition, a sizable portion must be kept in reserve for future lots, so the supply of these materials is nearly always limited. Finally, this can be difficult when the standards are unstable and/or not able to be frozen.

15.2.3 Secondary

Secondary standards are reference materials that are calibrated against a primary standard. These types of standards are created as a working standard so the primary standard supplies are not consumed. The source material is made in-house and should be linked to the primary standard. As stated previously, reduced characterization is needed since it is linked to the primary standard. Assuming there is an active production process, there can be a relatively limitless supply of material suitable for a secondary standard. This limitless supply is the issue since long-term variability is a concern. As long as the secondary standard stays closely linked to the primary standard, there should be no standard drift over time.

15.2.4 Laboratory

The final type of standard is a laboratory standard. This type of standard is a minimally characterized material purchased just as any other reagent is purchased. The requirement for minimal characterization is a distinct advantage. Unfortunately, this is also a distinct disadvantage since the standard is dependent on the certificate of analysis and the accuracy of the data contained on the certificate of analysis (CoA). Since this material is made by another company, there is a seemingly limitless supply. Since there is little to no characterization in the laboratory, there is little to no defendable data available if the validity of the standard is questioned. This type of standard should be avoided except in the simplest of assays and only when no higher level of standard is possible.

15.3 Process

The process for standard characterization is shown in Fig. 15.2. The process is described in detail in the section below.

15.3.1 How to Characterize a Standard

The first step in the characterization process is to write a protocol. This is useful even in non-regulated groups since this will outline the assays used to characterize the standard, prepare timelines, and determine the resources needed to generate the data. In regulated groups, this may require several different approvals. Once the protocols are approved, the material can be manufactured. (Note: It is acceptable for

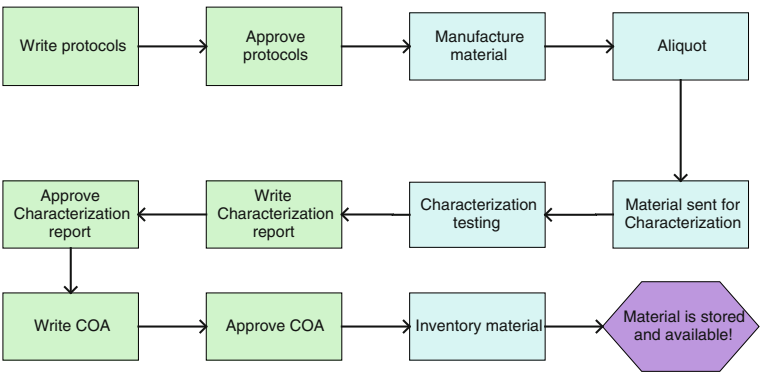


Fig. 15.2 Standard characterization process

the material to be made prior to protocol approval.) In general, standards should be aliquoted into individual use vials. This prevents contamination, provides a convenient presentation for the analysts, and makes usage and replacement determinations easy. Once the material is aliquoted, it can be characterized in the different laboratories. All aspects of the characterization must be closely monitored. It is important that the characterization utilizes "all the best." In many vaccine laboratories, standard characterization takes second priority to sample analysis. In laboratories which do not value standard characterization, the analysts used for characterizing the standards are those freed from sample analysis (usually because they exhibit more variability than other analysts or are not as skilled at the assays) and the instrumentation utilized are those which are not being used for sample analysis (again, because the instrument generates more aberrant data). Since these data are going to serve as the value to which all data are compared, the best analysts and the best equipment should be used for the characterization studies. There is an alternative opinion on this point. If there are multiple different laboratories, equipment, and analysts using the standard, then a study can be designed to incorporate all of the different variables. If this type of study is undertaken, it is important to do multiple replicates (e.g., design the study statistically) to ensure sufficient replicates are generated to understand the differences. Once all of the data are generated, a report summarizing the data is generated and approved. Any issues encountered during the characterization study should be reviewed and resolved in the final report. Once the report is approved, a CoA is prepared and approved. The standard is now approved for use.

15.4 Case Study—Human Gonadotropin (hCG) Reference Reagents

The World Health Organization (WHO) provides standards for some reagents such as human gonadotropin (hCG), which is used in pregnancy tests (Birken et al. 2003). Although not a vaccine example, there are useful concepts to be learned from the characterization of hCG reference reagents. Previous preparations have contained hCG metabolites as well as protease activities, making the reagents less effective and useful. A working group was created to characterize new materials for use as reference reagents.

The characterization of the reference reagents utilized sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) to determine the purity, isoelectric focusing (IEF) as an orthogonal purity measurement and to determine the isoelectric point (pI), Western blot as an identification technique, N-terminal sequencing as an orthogonal identification technique, mass spectrometry (MS) for molecular weight determination, high-performance liquid chromatography (HPLC) for additional

purity measurements, carbohydrate analysis for content, and amino acid analysis (AAA) for protein content.

SDS-PAGE, Western blot, HPLC, and IEF all showed the expected profile. The IEF should six major bands around pI = 4.5. The N-terminal sequencing showed less than 2–3 % contaminants. The MS showed hCG with a molecular weight of 37.5 kD. The carbohydrate Analysis agreed with expected content (11.1 % sialic content by weight). The AAA determined the protein quantity.

The conclusion of the characterization was that a highly purified, well-characterized reference standard for hCG was available for use. No significant contaminants and negligible protease activity was observed during the characterization.

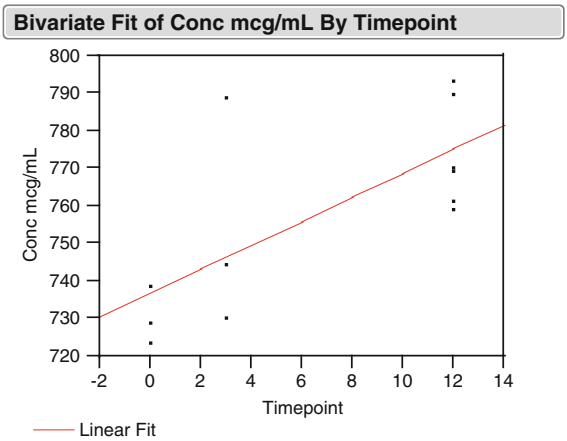


Fig. 15.3 Concentration over time with a linear fit applied

Linear Fit

Conc mcg/mL = 736.84412 + 3.1890174 Timepoint

Summary of Fit

RSquare	0.509571
RSquare Adj	0.460528
Root Mean Square Error	18.35607
Mean of Response	758.37
Observations (or Sum Wgts)	12

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	3500.9678	3500.97	10.3903
Error	10	3369.4532	336.95	Prob > F
C. Total	11	6870.4210		0.0091

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	736.84412	8.524932	86.43	<.0001
Timepoint	3.1890174	0.989333	3.22	0.0091

15.5 Case Study—Vaccine Stability Issue?

Stability issues can ultimately doom a vaccine. In this case study, there appeared to be a stability issue with the vaccine. A trend in the concentration over time was observed (Fig. 15.3). In addition, an out of specification (OOS) result on the vaccine candidate concentration triggered an investigation. The investigation tried to determine if the OOS is a manufacturing issue, an analytical issue, or a sampling issue?

In Fig. 15.3, a linear fit applied. The correlation coefficient is fairly poor due to variability in the data, but the correlation shows a statistically significant trend. However, the direction of trend does not make sense. The concentration should not be increasing over time. This would seem to rule out a manufacturing or sampling issue.

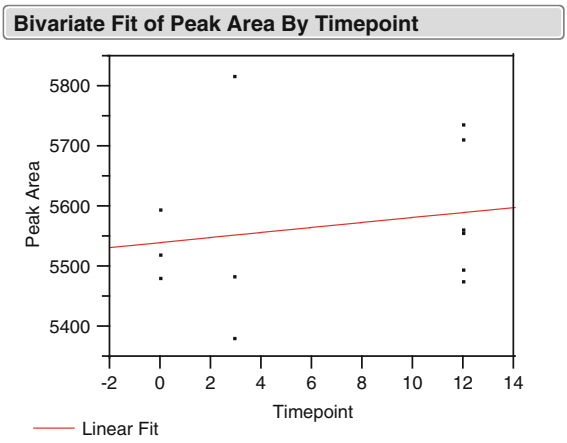


Fig. 15.4 Peak area over time with a linear fit applied

Linear Fit

Peak Area = 5540.0383 + 4.2591214 Timepoint

Summary of Fit

RSquare	0.035215
RSquare Adj	-0.06126
Root Mean Square Error	130.8001
Mean of Response	5568.787
Observations (or Sum Wgts)	12

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	6244.74	6244.7	0.3650
Error	10	171086.72	17108.7	Prob > F
C. Total	11	177331.46		0.5592

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	5540.0383	60.74624	91.20	<.0001
Timepoint	4.2591214	7.049705	0.60	0.5592

In order to determine if the issue was related to the material or the analytical measurement, the peak areas for the HPLC were plotted for the study and a linear fit was applied (Fig. 15.4). This model showed no correlation and no statistically significant trend (e.g., no change in peak area over time). If the concentration was truly increasing, why is peak area stable? The peak areas should be growing.

When this type of behavior is observed, it is often a standard issue. In order to determine if there was a change in the standard, the peak area of the standard whose concentration is closest to the samples was plotted. The data are shown in Fig. 15.5.

A key assumption was made at this point. If the UV lamp for the HPLC was stable, then the peak areas could be plotted over time. Once a linear fit was applied to the standard peak area, everything became clear. The issue was a change in the standard peak area over time. The R^2 (0.997) is excellent (although the data are limited). The data indicate an issue with the reference material. This is concluded from:

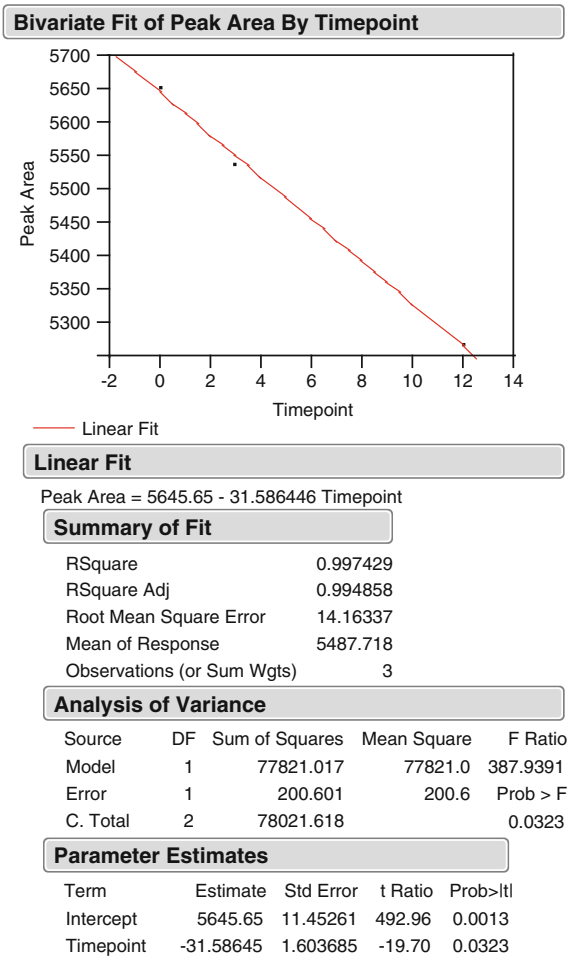


Fig. 15.5 Peak area for standard over time with a linear fit applied

- Statistically significant increases over time in sample concentration of the vaccine without a corresponding increase in peak area for the samples.
- Statistically significant decreases over time in the standard which corresponds to the peak area for the samples.

A decrease in the reference material would result in an increase in the sample concentration, unless the peak areas of the sample were also decreasing. However, the peak areas of the samples did not show a trend. Upon further investigation, the standard was a dry powder which was known to be hygroscopic. Over time, the standard was picking up water. This means some of the weight attributed to the vaccine was actually just water and was not going to contribute to the peak area. As expected, there was no stability issue with the vaccine.

Reference

Birken S, Berger P, Bidart J-M, Weber M, Bristow A, Norman R, Sturgeon C, Stenman U-H (2003) Preparation and characterization of new WHO reference reagents for human chorionic gonadotropin and metabolites. Clin Chem 49:144

Chapter 16
Lot Release of Vaccines by Regulatory Authorities and Harmonization of Testing Requirements

Rajesh K. Gupta, Chander K. Gupta and Laurent Mallet

16.1 Introduction

Unlike most drugs that contain chemical substances with known structure and purity, biological products, including vaccines, are made or derived from biological sources, such as cells, tissues, or plasma from humans, animals, or microorganisms. Often vaccines are complex products that cannot be characterized fully by physicochemical methods routinely used to characterize most drugs. Activity (safety and potency) of biological products is often measured by bioassays using biological systems, such as animals, tissues, and microorganisms. Therefore, most of the tests for safety and potency have large inherent variability due to biological nature of the product itself as well as variability of biological systems used in these tests.

To have an assurance of potency and safety of biological products, particularly vaccines, regulatory agencies around the world require an independent review of critical manufacturing and testing data, and an independent testing at the regulatory agencies for critical attributes for safety and potency before marketing each lot or batch of the product after licensure. This has been referred to as lot release (Health Canada 2005; World Health Organization 2013c), official release (US FDA 2013a) or official control authority batch release (OCABR) of biological products (EU 2013; EDQM 2013). Lot release has been an essential component of regulation of vaccines to (1) assure the acceptable quality and safety of each manufactured lot; (2) to obtain confidence in the strength of active components assigned to a particular lot, and (3)

R.K. Gupta (✉) · C.K. Gupta
Biologics Quality & Regulatory Consultants, LLC, 17821 Stoneridge Dr.,
North Potomac, MD 20878, USA
e-mail: rgupta@biologicsqualityandregulatory.com

L. Mallet
Sanofi Pasteur, 1541 Avenue Marcel Mérieux, 69280 Marcy L'Etoile, France

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6_16

to assess the validity and accuracy of the safety and potency tests performed on a lot by the manufacturer.

It should be noted that lot release is not an alternate to other regulatory activities. It is an important component of the total regulatory framework, including regulatory review and oversight during development and licensure of the product, regulatory inspections during review of an application and periodic good manufacturing practices (GMP) inspections, postmarketing surveillance (PMS), etc.

Differences in testing requirements in various countries cause complexity and delays in manufacture and release of quality products at affordable cost. There is a need for harmonization of global testing requirements and lot release of vaccines.

16.2 Background on Lot Release of Vaccines

The World Health Organization (WHO) (2013c) strongly recommends an independent lot release by national regulatory agencies for vaccines used in their country, at least by review and approval of the manufacturers' summary protocol, or through recognition of the decision of another regulatory authority.

In the US, this is required as per 21 CFR 610.2, referred as "Official Release" (US FDA 2013a) and in the Europe this is mandated through European Union (EU 2013) and European Directorate of Quality of Medicine (EDQM 2013) as OCABR, managed through the network of Official Medicine Control Laboratory (OMCL) network. Certain biological products, such as therapeutic proteins and monoclonal antibodies developed during approximately last 2 decades, have been exempt from this requirement in the US (Federal Register 1995) and other countries, because these products could be characterized by physicochemical methods. Such products are usually referred to as "well-characterized biological products." None of the vaccine products are considered as "well-characterized biological products" and are not exempt from the lot release requirement. However, on a case by case basis, certain products may not be subject to a routine lot release requirement. This depends upon the complexity of the product, consistent track record in manufacturing and testing and the history of the manufacturer with regulatory compliance. In the US, this is referred to "Alternate to Lot Release" or "Surveillance" (Federal Register 1993). Most of the vaccine products are not under "Alternate to Lot Release" and are subject to a typical lot release.

16.3 Why Is Lot Release Necessary for Vaccines?

Among biological products lot release is critical for vaccines, because vaccines are used in healthy normal populations. If strength of active components (potency) in a lot or batch of a vaccine is suboptimal, impact of using such a suboptimal lot will not be known for a very long period (years), because benefits of vaccination against a disease

are not visible immediately and the protection is not on an individual basis. Similarly, if there are safety issues with a particular lot or batch of a vaccine, the impact will be devastating as a large number of healthy normal individuals (whole populations, usually pediatric population) receive vaccines. Often the adverse effects due to safety issues, such as effects due to residual live virus or residual or reverting toxins, may not be known immediately (within a few hours or even days) after injection. During "the Cutter Incident" in 1950s affecting about 40,000 children with headaches, neck stiffness, muscle weakness and fever, including about 200 children with severe and permanent paralysis and 10 deaths, symptoms due to presence of residual live virus in the inactivated polio vaccine were not observed in children for several days after vaccination (Nathanson and Langmuir 1963a, b). By the time consequences due to safety issues or symptoms due to side effects after vaccination are visible or known, the lot or batch of the vaccine is already used due to mass vaccination campaigns as vaccines are given to large populations of healthy subjects, often during mass vaccination campaigns, to prevent transmission of the disease. Normal healthy populations take vaccines with a certain degree of risk of adverse reactions, based on the trust that regulatory agencies have high degree of assurance of safety and potency of these products. Lot release of vaccines by regulatory agencies provides such an assurance. When regulatory authorities do not perform a lot release as per the intent of this process, they violate the public trust in vaccines.

Further, importance of lot release for biological products cannot be undermined, because reference standards used in testing of biological products, including vaccines, may not be fully characterized and have the same issues of complexity and stability as the biological products, thus affecting the assurance of content in these products. Additionally, for most new products national or international standards and reference preparations are not available and there is limited data on the stability of in-house or working standards. Independent review of data from each lot or batch of a vaccine by regulatory agencies, including data on the performance and validity of these reference preparations during official or lot release of the lot is necessary to gain confidence in results of safety and potency tests used for release of the lot. In the beginning of twentieth century, significant efforts were made in establishment of international standards for biological products by Paul Ehrlich and Sir Henry Dale (Burn 1950). These efforts, particularly for vaccines, were continued through the League of Nations and the WHO's Expert Committee on Biological Standardization (ECBS) (World Health Organization 2004), Staten Serum Institute in Copenhagen, Denmark, US FDA's Center for Biologics Evaluation and Research (CBER), UK's National Institute for Biological Standards and Control (NIBSC) and International Association of Biological Standardization (IABS). Experts from these organizations and other vaccine manufacturing and regulatory institutions, most of whom were members of the ECBS and the IABS met regularly to discuss creation of International Standards for vaccines and other biological products. Scientists and reviewers in regulatory agencies performing lot release and other review activities gained critical knowledge about the challenges in manufacturing and testing of vaccines and discussed these in a proactive and scientific manner during various meetings. Strong biological standardization program managed through ECBS with scientific

discussions at the IABS meeting has been partly responsible for success of complex vaccines used during twentieth century in controlling many infectious diseases.

However, in the last 2–3 decades, vaccine industry has changed significantly, good for the most part, with regard to implementation of current good manufacturing practices (cGMP) regulations, and upgradation of manufacturing facilities to modern standards of clean rooms with classified environment. However, due to budgetary constraints at the WHO and many regulatory agencies, there has not been much effort to generate standards for new biological products, including vaccines developed during last 2–3 decades. During this period, the IABS also changed its priorities and focus from biological standardization to biologicals. These developments led to regulatory agencies relying solely on the manufacturers of these products for the reference materials. Often these reference materials are not independently calibrated or managed by the regulatory agencies. In such a situation, manufacturers did not have much option, but to generate and calibrate product specific homologous in-house reference materials for testing and releasing their products. Occasionally such reference materials have been referred as "Standards" due to confusion between terminology of "Standards" and "Reference Preparations" (Gupta 2011). Such preparations should not be referred as "Standards" as these have not been independently verified by a third party, such as a regulatory agency, for example, FDA, European Medicines Agency (EMA), or a standard management organization, for example, United States Pharmacopoeia (USP), EDQM, NIBSC or National Institute of Standards and Technology (NIST). In the broader principles of biological standardization, use of in-house reference materials, occasionally referred as "Standards" is a "conflict of interest" for manufacturers to generate, calibrate, and use standards for their products themselves without independent verification. There is a need for regulatory agencies, such as CBER, EMA, international bodies, such as WHO and standard management organizations, such as NIBSC, EDQM, USP, NIST, to facilitate independent calibration of reference materials and standards for new products. In the current situation due to absence of official "Standards" for new products, lot release process gets additional significance to assure safety and quality of new vaccines, because regulatory agencies get an opportunity to use manufacturers "reference materials," or "standards" during lot release testing. Additionally, regulatory agencies review data and performance of these "standards," when these were used by the manufacturers during release testing of the lot. Standards and reference materials have critical role in assuring quality of biological products, particularly for new combination vaccines, such as multicomponent vaccines.

16.4 Lot Release or Official Control Authority Batch Release Process

Currently, lot release of vaccines is done by (1) review of the manufacturer's protocols, usually referred to as lot release protocols (LRP) containing manufacture's information and results on manufacture and testing of a lot, and independent

testing at the regulatory agencies (full or selected testing) on all or selected lots, (2) review of LRP only, (3) alternate to lot release or surveillance, and (4) acceptance of lot release certificates from other regulatory agencies (Table 16.1). These approaches have been used for different products in the same country. There is a tremendous variability or heterogeneity in the lot release approaches in different countries, with EU performing lot release testing on 100 % of the lots released, whereas other countries releasing many vaccines based on review of manufacturer's LRP only, with no testing. It is possible to use various approaches in a same country for different products based on nature of the product, the postmarketing experience, including production history, safety profile (risk) of the product, manufacturer's track record with regulatory inspections and the availability of other independent evidence of product quality.

In general, when a vaccine is manufactured and licensed in a country, either for domestic use or for export, the regulatory authorities of the country take the responsibility for quality and regulatory oversight, including lot release. However, almost all countries importing the product perform an independent evaluation of license application, and postlicensure lot release of the product. Of course, the regulatory agencies of the country manufacturing the product share information with the WHO and the importing country depending upon the confidentiality and other agreements between regulatory agencies of manufacturing and importing

Table 16.1 Various approaches for lot release of vaccines

Approach	Application	Strengths/limitations
Protocols review and testing	New products Complex products Combination vaccines	Best option, when large fractions (>25 % lots) tested Reviewers gain product knowledge Require resources and expertise Require quality management systems (QMS)
Protocols review only	After significant knowledge about product Products with precise and accurate methods	Does not requires significant resources Does not provide independent evaluation of quality of the product Trending and tracking of manufacturers' results critical
Alternates to lot release/ surveillance	Characterized product Good track record of quality of product Manufacturer with compliance	Better use of resources Decision should be made after reviewing benefits and risks
Acceptance of lot release certificates from other agencies	Confidence in QMS of agencies performing release	Mutual recognition avoids unnecessary testing

countries. For global manufacturers, lot release performed by multiple countries independently sometimes with different testing methods and specifications, cause extra burden with regard to performing multiple tests for the same quality attribute and also with regard to cost and time in making products available in many countries at an affordable cost.

In Europe, mandated by the European Commission, EDQM is overseeing OCABR network. It is a specific network within the General European OMCL network. This network is covering vaccines, blood and plasma derivatives. As described in the EU administrative procedure for OCABR (EU 2013), the following principles are followed: "For batches of a medicinal product to be marketed in a Member State requiring OCABR, there shall be an Official Control Authority Batch Release Certificate common to all Member States. This shall show that the batch of medicinal product has been examined and tested by an OMCL within the European Union (EU) in accordance to this procedure and with OCABR guidelines pertaining to the medicinal product within the EU and is in compliance with the approved specifications laid down in the relevant monographs of the European Pharmacopoeia (Ph. Eur.) and in the relevant marketing authorisation (MA)."

The Member State informs the Marketing Authorization Holder (MAH) that its authorized human biological medicinal product is subject to OCABR. Samples of the batch to be released are sent, along with production and control protocols, to an OMCL within the European Union (EU)/European Economic Area (EEA). If the results are satisfactory, the Competent Authority (CA) issues an 'Official Control Authority Batch Release Certificate' to the MAH. This certificate means that the batch has been examined and tested by an OMCL in accordance with the OCABR guidelines pertaining to the medicinal product and is in compliance with the approved specifications laid down in the relevant monographs of the Ph. Eur. and in the relevant MA. The MAH must provide a copy of the OCABR Certificate to the CA of the Member State where the batch will be marketed. The certificate is recognized by all members of the network (EDQM 2013).

It is important to note that the MAH proposes OMCL(s) which would carry out OCABR for the product and that the collaboration with the OMCL(s) should begin at least 1 year before submission to allow for input to be considered in the development of suitable testing methodology for potency assays and batch release. Transfers of analytical methods and related critical reagents between the MAH and the OMCL(s) are usually part of this collaboration process.

The OCABR legislation concerns EU/EEA Member States and is also applied by any state having signed a formal agreement which includes recognition of OCABR, with the EU, via a Mutual Recognition Agreement (MRA).

Another specificity of the European legislation is the role of the Qualified Person (QP) in release of vaccines. As described in the Annex 16 "Certification by a Qualified Person and Batch Release" from EU GMP (European Commission 2001, under revision), each batch of finished product must be certified by a QP before being released for sale or supply in the EU/EEA or for export. The QP from the MAH will take personal responsibility for this certification by ensuring that the batch has been manufactured and tested or evaluated in accordance with the

requirements of MA, the principles and guidelines of EU GMP or the GMP regulations of a third country recognized as equivalent under a MRA. If products are imported from a third country, and if no MRA is in operation between the European Community (currently called EU) and the third country, samples from each batch should be tested in the EU/EEA before certification of the finished product batch by a QP. This is what is commonly called "Testing on Importation" (TOI).

To give an example, it is interesting to note that a vaccine lot manufactured (totally or partially) in the US destined for sale in US and EU is release tested (e.g., for potency) by the US manufacturer, the FDA, the importing manufacturing company in the EU and the OMCL in EU.

16.5 Lot Release Protocols

Since protocol review is an essential component of the lot release process, it is critical to develop carefully the template of the LRP based on the approved license application or MA. LRP templates for a number of vaccines are available as part of the WHO Guidelines for many vaccines, which can be a good starting point for a country-specific LRP (World Health Organization 1990, 1994, 1999, 2005, 2011a, b, c, 2013a, b, d, e, f, g). The format and the content of manufacturers' summary protocols or LRP that will contain important information taken from the production and test records for ensuring quality of the vaccines are generated during review of license application by the regulatory agencies. LRP must contain information on the traceability of critical starting materials, such as seeds and cell banks, active and critical components used in the manufacture of the product, and the results from tests performed by the manufacturer at various stages of manufacture, including tests performed on critical components, intermediates, final bulk, and final product. Careful attention should be paid to include critical information on tests for safety, potency, and quality performed at various stages of manufacture. The LRP should also include information on standards and internal controls used in the test to demonstrate the performance of these materials and validity of the test. In practice, manufacturer submits a LRP template along with the license application and works with the regulatory agencies to finalize the LRP template that should include important manufacturing and testing summary required to evaluate quality of the product. The protocol may need to be amended when changes are made to the approved production process, testing methods and specifications. Of course, these changes are approved by the regulatory agencies.

LRP is product specific, but basic structure of the LRP containing a number of general items should be similar to achieve consistency and ease of reviewing. This information includes details about the manufacturer, lot number and size, manufacturing and expiration dates, genealogy of the manufacture of a lot (flow chart) with traceability to components used and testing performed at various stages of manufacture, summary of various stages of manufacture starting with seed strains and cell banks, and testing performed at various stages with specifications (Table 16.2).

Table 16.2 General information typical for a lot release protocol

Information
Details about manufacturer, including name, location, and license number
Name of the product, lot number, and size of the lot
Dates of manufacturing and expiration
Flow chart showing genealogy of the lot with traceability to components and testing performed at various stages
Summary of each manufacturing stage with testing performed
Summary of final bulk (formulation)
Tests performed at various stages with date of test, method, summary, and performance of critical reagents, including reference preparations, standards, internal controls, summary of assay validity criteria (for example, slope, intercept, linearity, 50 % end points, results of internal controls, challenge doses)

In Europe, at the time of submission, the OMCL(s) eligible for OCABR, in close collaboration with the Rapporteur and Co-Rapporteur, indicate the need to prepare an appropriate guideline to the relevant OCABR drafting group and/or the OCABR Advisory group. This includes a list of tests to be performed during OCABR and highlighting of any special issues related to the model protocol. The drafting group determines if a new guideline is needed or if an existing guideline can be revised. The draft OCABR guideline/protocol should be ready at the time the MA is granted and is formally adopted by the OMCL batch release network once the MA has been finalized.

16.6 Lot Release Testing

Testing at regulatory agencies for lot release of vaccines, particularly immediately after licensure of the product and for complex vaccines, plays a critical role in monitoring quality of the products and the consistency in manufacture. Such testing also enables regulatory authorities to gain scientific and technical knowledge about the testing methods in collaboration/support with the manufacturers. Technical expertise developed at the regulatory agencies helps in assessing various issues, including changes in specifications, methods, etc., which are key to maintain quality of the product. Maintaining expertise in testing at regulatory agencies is important for the competence of these agencies in effectively monitoring the product.

In addition to verifying compliance to the specifications and the test results of manufacturers, lot release testing also provides an assurance on the continuing suitability of the methods and acceptable performance of the manufacturer's generated reference standards and internal controls. Due to regulatory agencies' knowledge and experience with the testing methods, it is easy to implement better and modern methods for testing. Further, the expertise in testing is useful during periodic inspections of the manufacturing facilities and quality management systems (QMS) used during manufacture and testing of the product.

For lot release testing to be effective and meaningful, the laboratories at the regulatory agencies should have adequate resources and should operate under a QMS. Testing performed without adequate resources and without a QMS may generate inaccurate results leading to delays in release of products or rejection of quality products.

Testing strategy or plan needs to be established, including tests to be performed at the regulatory agencies and frequency of testing. Testing strategy for a product is developed, when regulatory and scientific review of testing methods, including performance of safety and potency related tests are completed at the regulatory agencies during evaluation of license application. A risk-based approach in selecting the methods to be performed during lot release testing and in developing frequency of lots to be tested would optimally utilize resources at regulatory agencies.

There is a wide variability in the percentage of lots of a product tested by various regulatory agencies around the world, with Europe testing 100 % of the lots and other regulatory agencies performing no testing for a number of products. For new licensed vaccines and complex vaccines, particularly those without a surrogate potency test for clinical efficacy, large fractions of lots should be tested to assure quality of vaccines and their consistency in manufacture. When a reduced testing is performed due to inadequate resources, lots selected for testing should be representative of the total production. Assuming the product is stable at this stage, testing final bulk may represent more final product lots, because multiple final lots are usually made from a final bulk. The testing plan, including tests performed and the percentage of lots tested, should be evaluated periodically and may be revised on the basis of results from lot release testing. A good consistency over a period can reduce percentage of lots tested, whereas repeated failing results and/or specific testing issues may increase percentage of lots tested.

When regulatory agencies do not have adequate resources or testing laboratories, lot release testing could be performed by a contract testing laboratory with appropriate confidential disclosure agreement and having adequate technical and regulatory capabilities. The contract testing laboratory should be inspected or audited for the technical capabilities and the laboratory QMS.

In Europe, the OCABR procedure consists of a critical evaluation of the manufacturer's production and control protocol, and of the testing of the samples submitted by the manufacturer as specified in the relevant guidelines.

Regulatory agencies, particularly those with inadequate resources and not performing much testing, should consider lot release testing for new vaccines and for products from new manufacturers for which there is little accumulated experience.

16.7 Selection of Methods for Lot Release Testing

Due to limited resources at many regulatory agencies, testing methods should be carefully selected, choosing those methods that add value to the assurance of safety and potency of vaccines. Testing for all product release parameters may not add

much value. Test methods that ensure safety and potency of products during lot release testing include potency, purity including free saccharide components of the polysaccharide-protein conjugate vaccines, specific toxicity tests, and other product specific tests such as adsorption of vaccines onto adjuvants, appropriate strains in the product (strain identity), etc.

It is also important to carefully select the stage at which lot release testing should be performed. Final product or drug product is a stage where most testing is desirable. However, it is not possible to perform all quality related testing at the final product. Certain vaccines, such as influenza vaccines in the US, are released at final bulk stage in order to have these products available faster for use. Certain complex vaccines, particularly combination vaccines, may require testing at intermediate stages, such as monovalent bulks or individual vaccine components for complete evaluation of quality and safety. Testing at intermediate stages may also be necessary when the final bulk and final product cannot be tested due to the presence of adjuvant in the final product. For influenza vaccines, seed virus of the strains used for a particular season, may also be tested by regulatory agencies.

In Europe, the selection of the methods happens at the time of the MA grant. The tests are selected by the OCABR drafting group on the basis of the dossier submitted by the MAH. Normally OCABR consists only of phase 1 testing described in the guideline. However, in some cases (e.g., a significant change in the manufacturing process, adverse events, unexpected variability in the results of quality control tests performed by the manufacturer or the OMCL, a critical inspection report from the medicinal inspectorate), phase 2 testing may be appropriate as a transitory measure. Phase 2 testing represents a set of additional testing measures.

Some quality attributes may not add value for lot release testing and should be monitored through other tools. Sterility and general safety tests (GST) that require aseptic testing laboratory and animal facilities, respectively, are examples of tests that do not add much value to lot release testing and should not be performed at the regulatory agencies.

16.7.1 Sterility Testing in Lot Release

Sterility test, performed to detect microbial contaminants, is a requirement for all injectable drugs that cannot be terminally sterilized. Most biological products, including all vaccines, cannot be terminally sterilized. These products are manufactured aseptically and tested for sterility at various stages of manufacture. Sterility test does not demonstrate absolute sterility of the product, but is one of the components along with aseptic process validation, environmental monitoring and use of sterilized equipment and reagents, providing assurance that biological products are free from detectable microbial contaminants and meet sterility requirements, specified in the US FDA requirements for biological products (US FDA 2013c; USP 2011; European Pharmacopoeia 2013a).

As emphasized earlier in this chapter, vaccines are used in healthy subjects, including babies and must have the highest degree of assurance for absence from microbial contaminants. Sterility test, performed during various stages of manufacture of vaccines, is important component to assure sterility of the product as well as to assure validity of aseptic manufacturing process. However, testing small fraction of final product at regulatory agencies for sterility test does not add any value. Instead, sterility test requires significant resource in terms of need for an aseptic testing laboratory with environmental monitoring. Further the test takes 2 weeks causing delays in the lot release and may cause further delays due to false positives, which occur at a higher frequency if the test is not performed in an isolator.

16.7.2 General Safety as a Lot Release Test

GST was started on biological products during early part of twentieth century after death of individuals taking products containing extraneous toxic substances. As per US FDA Code of Federal regulations (2013b), most biological products for administration to humans, including vaccines, must meet the requirements of GST, unless these products have been exempted from this test by the regulations or FDA (Federal Register 1998a, b). The GST is intended to help ensure the safety of such products by detecting extraneous toxic contaminants that may be present in the product at the final container. The test is required for every final product lot of the biological product. Such toxic contaminants may be bacterial and fungal by-products that persist after the bacteria are removed by filtration or killed by sterilization, or formulation errors that result in harmful levels of certain substances, e.g., preservatives. The test serves as a safety net to detect harmful contaminants.

Over the years with advances in testing methods and the implementation of cGMP regulations in manufacture of vaccines during 1990s, GST lost its utility, particularly in the presence of product-specific toxicity tests, such as specific toxicity test for tetanus and diphtheria products, mouse weight gain test for whole cell pertussis vaccine and inactivation or residual infectious virus test for inactivated viral vaccines (Gupta 1996). Abnormal toxicity test which is equivalent to the GST was eliminated from the European Pharmacopoeia (Ph. Eur.) for vaccines due to inherent toxicity of certain vaccines, which interfered with the GST and also due to the fact that no vaccine product ever failed the GST or abnormal toxicity test (Schwanig et al. 1997). In the US, the FDA published in the Federal Register, a final rule exempting certain biotechnology and synthetic biological products from a number of regulations applicable to biological products, including the GST (Federal Register 1998a, b).

To overcome the inherent toxicity of such vaccines in the GST or abnormal toxicity test, the test had been modified, so that the product passes the test (Bhandari et al. 1990; World Health Organization 1990). Modification of GST compromised the original intent of the test to assess the vaccine for any toxicity at a single human dose. The important aspect in removal of the GST is to understand the inherent toxicity

observed in GST or other preclinical toxicity testing and to develop appropriate product specific tests to manage and control inherent toxicity due to product itself. Product specific toxicity tests have significantly different test design as compared to abnormal toxicity or GST in terms of choice of animal species, dose and observation period, to detect the inherent product toxicity. For example, Specific toxicity test for diphtheria vaccine uses five guinea pigs injected with 5 times the human dose and observed for 6 weeks, whereas abnormal toxicity test used 2 guinea pigs and 5 mice and GST uses 2 mice and 2 guinea pigs that are observed for 1 week only. The reason not to use mice in the specific toxicity test of diphtheria vaccine is that mice are not sensitive to diphtheria toxin that may be present in the vaccine (Gupta et al. 1997). The longer observation period for specific diphtheria toxicity test is to detect possibility of reversion of toxoid to toxin, which can happen in the body at 37 °C (Akama et al. 1971). GST is not suitable for such purposes. It should be noted that need for the specific toxicity test should be evaluated case by case depending upon the inherent toxicity of the product. For many recently licensed vaccines, comprising purified components, specific toxicity tests are not required.

For a new biological product, it is important to understand inherent toxicity of the product during preclinical and clinical development, particularly the final formulation given to humans. For certain vaccines, adjuvant and the vaccine product are mixed before use. It is important to evaluate toxicity of the mixture in an appropriate product specific toxicity test, if required, that is suitable to detect toxic reactions of final formulation. GST is not a scientifically sound test to detect product specific toxicity.

16.8 Quality Management Systems for Laboratory Operations at Regulatory Agencies

All lot release activities at the regulatory agencies, including review of LRP, must be performed under the QMS, appropriate for laboratory operations to ensure reliability of results and avoiding false “Out of Specifications (OOS)” results that may cause delays in release of lots and even rejection of quality products. The QMS system should comprise the following subsystems: sample management with full chain of custody of samples received, documentation, including written procedures and controlled worksheets to capture raw data during testing, training of personnel, equipment life cycle management, including qualification, preventative maintenance, calibration and validation of all equipment and instruments used in testing, analytical methods life cycle management, including method development/qualification, if required, method validation, method transfer, trending and tracking, internal and external audits, management of deviations, nonconformances, OOS, including investigations with a thorough root cause analysis, leading to effective corrective actions and preventative actions (CAPA) (Table 16.3). The testing laboratories should have an oversight by an independent Quality Assurance (QA) Department, ensuring the verification of appropriate QMS and their implementation.

Table 16.3 List of subsystems required for an effect quality management system (QMS) in laboratory operations for lot release testing

QMS subsystem	Element, important factors
Roles and responsibilities	Quality assurance group independent of laboratory operations
	Progression of responsibilities with positions
Training	General, safety, cGMP, on-the-job training
Documentation	Management and control
	Written procedures
	Controlled worksheets to record raw data
	Procedures for generation, documented review, correction/change of documents, data and records
	Procedures for security, audit trail and backup of electronic data and records
Sample management	Procedure for receipt, storage, testing and disposal of sample, including documented chain of custody
Equipment and instruments	Life cycle management of laboratory equipment and instruments, including written procedures for qualification, operation, monitoring, maintenance, calibration, verification, repair
Critical reagents, standards	Procedures for receipt, approval for use, assigning expiration, disposal, etc. of reagents
	Generation, calibration or qualification of standards and critical reagents
Analytical methods life cycle	Development, qualification, validation, verification, transfer
Change control	Documentation of changes to method
Deviations, OOS, nonconformances, and complaints	Written procedures for documentation, investigations, corrective action preventive actions (CAPA)
Investigations	Root cause analysis
CAPA	Correction, corrective and preventative actions
Trending and tracking	Important parameters of standards, internal controls, results
Internal and external audits	Independent audit of quality management systems
Vendor qualification	Qualification of vendors performing calibration, maintenance, providing critical reagents, or other services
Management review	Management review of performance of laboratory operations
Stability	Stability of critical reagents
Environmental monitoring	For controlled and classified environments

The WHO provides assistance and guidance to the national regulatory agencies in setting up the QMS (WHO 1992).

Adequate resources in terms of appropriately designed laboratories suitable for intended testing, qualified personnel with the appropriate technical and scientific expertise, qualified and well maintained equipment with appropriate calibration and

verification, and qualified reagents, reference standards and internal control are essential to generate reliable results during lot release testing.

QMS for review of LRP include appropriate checklists used in the review of protocols to ensure a complete and consistent review (WHO 2013c). Such checklists also ensure appropriate documentation of the review. Tracking and trending of manufacturer's test results, including performance parameters of standards and internal controls from LRP along with data from lot release testing performed by regulatory agencies are essential components of QMS in ensuring quality of the product and in achieving consistency in manufacture.

16.9 Setting up Testing Methods at Regulatory Agencies

With adequate technical capabilities, laboratory space, appropriate equipment and independently accredited QMS, regulatory agencies can set up test methods that will generate reliable results. For setting up test methods for lot release efficiently, the regulatory agencies should plan transferring methods from the manufacturers during late stages of clinical development of the product or during licensure process, including setting up and appropriate qualification and validation of the methods. These method transfers are not necessary for compendial tests.

16.10 Conducting Lot Release

The manufacturers' LRP must be carefully reviewed by the regulatory agencies before release of a lot for use to ensure that the lot met all specifications and the test methods met all validity criteria with acceptable performance of standards and internal controls. Review of LRP by the regulatory agencies should be appropriately documented with selected data tracked and trended to assess product consistency. Additionally, independent testing should be performed for critical safety and potency attributes, particularly for legacy complex vaccines and newly licensed vaccines. When marketing products in multiple countries, results of testing performed at regulatory agency of the manufacturing country or other countries may be provided to avoid repetitive testing. Information about such testing or the lot release certificate from another country can be provided with the LRP.

16.10.1 Protocol Review Process

The quality and value of the LRP review depend on the information provided by the manufacturer in the protocols. Review of LRP requires sound technical and regulatory knowledge of the product and analytical methods. The LRP for a product can

be reviewed by a subject matter expert for that product or by a team of experts depending on the complexity of the product. A validated software with appropriate access control, security, and an audit trail for tracking and trending of the data from LRP is essential for performing a meaningful review of protocols.

The lot release process starts with receipt of the manufacturers' protocols and test samples. The receipt of LRP and samples are appropriately documented by the regulatory agencies, after verification of the label information for the test sample and on the protocol. Next, it should be confirmed that appropriate and correct version of LRP has been used by the manufacturer. Then the protocols are routed to predetermined reviewers, who are subject matter experts by a manual system or by an online system. The review of LRP should be managed through an independently accredited QMS. Software used documenting review of LRP and tracking/trending of data should be appropriately validated. Captured data should be reviewed periodically for any trends or atypical observations. Such findings should be discussed with the manufacturer to take appropriate corrective and preventive actions, if necessary to ensure quality of products and consistency in manufacture.

16.10.2 Capturing, Tracking, and Trending of Data for Lot Release Process

Capture, tracking, and trending of appropriate data during lot release process are important to add value to the process. Results on safety and potency attributes of the product and other critical parameters from the LRP and from regulatory agencies testing should be tracked and trended with appropriate periodic review. In the absence of standards for new products, critical parameters of reference preparations should be tracked and trended to monitor performance and stability of these preparations over time. A trend or a shift in the slope, intercept or 50 % endpoint of a reference preparation may indicate a problem with the reference preparation or other critical reagents used in the test method. Validity criteria for various quality attributes of a reference standard or results of internal control are usually based on 95 or 99 % confidence intervals. Wide variations from expected 95 % or 99 % acceptance of that attribute indicate inaccuracy in setting the limits or performance of that attribute. Else the data are not normally distributed, which is usually the case with biological methods. Appropriate measures should be taken to change the limits.

16.11 Challenges in Lot Release

From manufacturers' perspective, when the same lot of a product is supplied to multiple countries, independent testing at various regulatory agencies is not only costly and time-consuming, it may cause regulatory complexities due to high inherent variability of many biological assays. Repetitive testing and lack of

experience at certain regulatory agencies with complex assays can result in a "false" OOS results which then require extensive investigation and can cause delays in vaccine supply. The decision to repeat tests on a lot that has already been tested by another competent authority should be carefully considered in light of all available information. While it is important to perform independent testing at regulatory agencies, it is necessary that the regulatory agencies have adequate resources with regard to equipment, scientific expertise, and independently accredited operational quality systems to perform laboratory operations. It is an issue with regulatory agencies in developing countries, particularly for complex assays or methods requiring expensive and modern equipment.

The development and implementation of better test methods in terms of accuracy, precision, and also better surrogates for human efficacy should be encouraged by regulatory agencies. However, due to fear of subjective and unpredictable response of regulatory agencies to atypical results or new information from new test methods with higher accuracy, sensitivity and precision, manufacturers have been reluctant in some cases to adopt new methods. Such methods may be highly beneficial for legacy vaccines to ensure not only quality and consistency in manufacture, but also providing information that may help in improving these products.

Overall, the implementation of alternative methods, including development and validation, is a long process requiring significant resources and a persistent approach at the level of the manufacturers and/or regulatory agencies. A formal cross-validation or demonstration of equivalency of the new or alternative method against the current or compendial method may be challenging because:

- Most of the compendial methods have been developed at a time when current validation requirements (International Conference on Harmonisation 2005) were not in place and these methods may not be validated to the current standards.
- In particular current in vivo potency tests, having high variability may not allow establishing a correlation with the proposed in vitro method with high precision, mainly due to low precision of the current in vivo method.
- The alternative method may detect one characteristic of the microorganism to be detected (e.g., nucleic acid) while the compendial test detects another characteristic (e.g., viable or infectious microorganism).

For these reasons, there is a need to reevaluate the way alternative methods are developed, validated by manufacturers and finally accepted by the regulatory agencies. Some ongoing or recent approaches are promising. For example, the global efforts between manufacturers of rabies vaccines for human and veterinary use, regulatory agencies, National Institutes of Health (NIH), and several organizations to replace the in vivo *potency* test (called "NIH test") used to release these vaccines by in vitro alternative methods consistent with 3Rs principles of animal testing (Replacement, Reduction and Refinement) (Stokes et al. 2012). For this particular test, a "concordance study" demonstrating the ability to discriminate between potent and subpotent lots with a qualitative comparison of the alternate in vitro method with the in vivo mouse challenge test rather than a statistically

significant correlation is recommended. This is mainly due to low precision or high variability in the in vivo mouse challenge test.

Another recent successful approach to implement alternative methods is the example of mycoplasma Nucleic acid Amplification techniques (NAT) where the guidelines for validation of the alternatives methods and for comparability study were published in January 2007 as part of the Chap. 2.6.7 from Ph. Eur. (European Pharmacopoeia 2013b). These guidelines provided clarity in the development and validation of alternative methods to the existing tests for mycoplasma detection and helped significantly in developing and validating alternative methods.

For the older vaccines, there are common release methods, most of which have been developed at regulatory agencies. With development of new vaccines during last 2 decades, test methods for same product, for example *Haemophilus influenzae* type b (Hib) conjugate vaccine, have been different for various manufacturers. This has been due to proprietary nature of the products, inadequate resources at regulatory agencies in developing new methods or harmonizing various methods and nondevelopment of standard and reference materials for new products by the regulatory agencies. This situation leads to having regulatory agencies perform a slightly different test method for lot release testing than the method approved for that product. Such scenario may cause delays in release of the product due to discrepancies in results between the manufacturer and the regulatory agencies. In this situation the regulatory agencies should perform the approved test method defined in the dossier.

Any testing of samples for lot release outside approved methods should not be performed without a defined goal of the testing and risk mitigation strategies, when atypical results or new information about the product is obtained.

16.12 Challenges and Opportunities in Global Requirements for Testing Vaccines

Testing requirements for licensed vaccines are described in several pharmacopeias around the world including European Pharmacopoeia (2014a), United States Pharmacopoeia (2013), Japanese Pharmacopoeia (2011), Chinese Pharmacopoeia (2010), and Indian Pharmacopoeia (2014). In addition, in the United States, general testing requirements for vaccines are described in the Code of Federal Regulations (e.g. US FDA 2013b, c). Prior to mid-1990s, 21 CFR 600 had sections for individual vaccines containing detailed requirements for testing licensed vaccines. With the development of manufacturer specific vaccines, for example, Hib conjugate vaccines, and changes in testing requirements to the licensed vaccines through supplements or changes to the licensed applications, regulatory authorities could not keep the testing requirements in 21 CFR 600 current. In the US, testing requirements for vaccines are those approved in the license application or supplement to the license application. However, in Europe, all marketed vaccines must meet, unless otherwise justified and authorized, the requirements in specific

monographs in Ph. Eur., which are regularly updated. To provide guidance to the industry and communicating current thinking on testing requirements, particularly for new vaccines, testing recommendations are provided in the FDA Guidance for Industry, EMA and ICH guideline documents. Further, WHO has published recommendations for almost all marketed vaccines as well as some vaccines under development such as vaccine candidates against Dengue (WHO 2013f).

Often these testing requirements, particularly those in individual monographs of 21 CFR 600 prior to mid-1990s, EP monographs and guidance documents include detailed descriptions on test procedures, including description of operating conditions as well as manufacturing stages where these tests should be carried out. This sometimes leads to specifying strictly the way the test must be performed, restricting the manufacturer to develop a product and host cell substrate specific tests based on the risk analysis. An example for such testing requirements is the adventitious agent testing required for cell banks and viral vaccines. Majority of these tests have been inherited from the tests developed in 1950s for primary rhesus monkey kidney cells used in the production of polio vaccines. Based on experience with the manufacture of polio vaccines, detailed guidelines and monographs are available for adventitious agents testing of cell substrates and viral seeds. Overall requirements and tests are well described and useful for the industry. However, subtle differences in testing required by different regulatory agencies, related to operating conditions, stage of testing or nature of the sample to be tested and suitability of all tests for well-characterized human diploid cells and continuous cell lines, add complexity and unnecessary testing burden for the products marketed in multiple regions.

Various regulations and guidelines for the adventitious agent testing are described in the following documents.

- FDA Guidance for Industry, "Characterization and Qualification of Cell Substrates and Other Biological materials Used in the Production of Viral vaccines For Infectious Disease Indications" (US FDA 2010) which is applicable to both cell substrates of human or animal origin and viral seeds, intermediates, live attenuated and inactivated viral vaccines as well as purified recombinant proteins.
- The Ph. Eur. Chap. 5.2.3 "Cell Substrates for the Production of Vaccines for Human Use" applicable to diploid cell lines and continuous cell lines used for the production of vaccines for human use (European Pharmacopoeia 2013c).
- The Ph. Eur. Chap. 2.6.16 "Tests for Extraneous Agents in Viral Vaccines for Human Use" applicable to viral seeds and harvests manufactured on human and animal cells and on eggs (European Pharmacopoeia 2013d).
- WHO recommendations on cell substrates "Recommendations for the evaluation of animal cell cultures as cell substrates for the manufacture of biological medicinal products and for the characterization of cell banks" applicable to animal cell substrates used for the productions of biological medicinal products including vaccines (WHO 2013h).

Some of these documents are Guidance documents with implied flexibility that equivalent or better methods suitable for intended purposes may be used. However, several subtle differences between these monographs and guidelines result into

differences in testing for the same product for different regions if the reviewers at regulatory agencies do not show flexibility. Despite the flexibility in the guidelines documents, subjectivity in reviews and in acceptance of alternate methods to those specified in guidelines results in having multiple testing profiles for the same attribute to meet the global regulatory requirements. For example, for the in vitro adventitious agents testing on cells, the Ph. Eur. Chap. 5.2.3 requires three tests (hemadsorbing virus test on cells, extraneous agent test on cell culture fluids and cocultivation of intact and/or disrupted cells), while the WHO guidelines recommends the testing to be combined in a single test using cells or cell lysate with spent culture fluid. For the same in vitro tests, FDA recommends using 3 cell types with an incubation for 2 weeks and a subculture for additional 2 weeks, while WHO guidelines and Ph. Eur. monographs require a minimum of 2 weeks incubation on at least 2 cell types.

US FDA may have a flexible approach at the time of submission, but the sponsor or manufacturer must have sound scientific data to support alternative method to that suggested in the Guidance document. As described in the FDA guidance for industry (US FDA 2010), "alternatives such as those recommended by WHO or EP or different inoculation strategies for testing cell bank lysates and viral harvests will be considered when justified in the context of the entire testing program." The best way to deal with subjectivity in regulatory reviews is submission of clear and convincing rationale for the alternative method supported by strong science. Additionally a sound risk analysis of the alternative method or approach with regard to the impact on the quality of the product will be helpful in having these methods or approaches approved by the regulatory agencies.

However, in some cases, the subtle differences are historical and the scientific rationale regarding these differences has been lost. For example, for the in vivo adventitious test in adult mice, the Ph. Eur. Chap. 5.2.3 requires i.m. and i.c. routes of inoculation while WHO and US FDA recommend i.p. and i.c. routes of inoculation. The same subtle difference is observed for the in vivo test in suckling mice where a s.c. route is required in Ph. Eur. Chap. 5.2.3 while and i.p. and i.c routes are recommended by US FDA. A similar situation is also observed for the in vivo adventitious agent tests in eggs where a passage is recommended after 3 days for the allantoic route and 9 days for the yolk sac route by the FDA, while for WHO and Ph. Eur. the observation period is not less than 5 days without passage. All these subtle and small differences, if not harmonized, lead to performing multiple animal tests for the same attribute to satisfy global requirements.

Due to legacy of the adventitious testing from the primary rhesus monkey kidney cells, certain tests may not be required for well-characterized cell banks. For example, test for Mycobacterium was required for the polio vaccines made in primary rhesus monkey kidneys due to high susceptibility and high incidence of tuberculosis in rhesus monkeys. Such testing does not have much scientific basis for well-characterized human diploid cells, cells lines and insect cells. The insect cells are not susceptible to Mycobacteria, but require this test. Similarly testing insect cells for Mycoplasma on Vero cells is not scientifically sound because Mycoplasma for plants, such as *Spiroplasma citri* does not infect Vero cells.

The adventitious agent testing package is one example but it is clearly not an isolated example. There are other examples of differences between various tests performed in different regions. For example, the abnormal toxicity test in Europe and GST in the US and other tests described in the regulations. Such differences have several consequences for global manufacturers of vaccines. These include:

- Complexity in worldwide registration or licensure of new products with impact on global vaccine supply and availability of new vaccines in a timely manner for some countries. This complexity is increasing with the expansion of some pharmacopeias (e.g., Chinese Pharmacopoeia) particularly if new requirements and/or different methods/specifications are included in these pharmacopeias.
- Slowing the development and implementation of new methods by manufacturers because proposed alternative methods must show equivalency to several methods considered as "compendial."
- Regulatory questions, observations or citations during inspections with regard to the status of "compendial nature" of the method because one method considered as "compendial" in one region may not have the same status in another region. Thus the inspector from the second region will require a full validation of the method. This has a huge impact, for example in Europe and the US, compendial methods are considered as validated per se and thus only the suitability of the method under the actual conditions of use should be demonstrated (European Pharmacopoeia, General Notices 2014b). If the inspector from another region does not recognize the method as a compendial, then the manufacturer will be cited with a deficiency of absence of an in-house method validation.
- Following several testing profiles to comply with various requirements, particularly in implementing post approval changes. The complexity in the batch release process and global supply is, of course, increased by these potential multiple testing profiles and/or specifications. This complexity is further increased by the submission of post approval changes to multiple regulatory agencies and approval of these changes at different times. Since this process is not harmonized across the world, manufacturers may be in a difficult situation when a change is approved by one regulatory agency, which then expects to have the change implemented immediately upon approval, whereas the change is still under review with another regulatory agency. This creates a situation where the manufacturer may be following two processes or performing two tests, the new or proposed for the market whose regulatory agency has approved the change and the current or old for the market where the change is under review. Finally, one country may require a modification of the test, additional validation data while the other countries have accepted the new method.

Based on these examples and in order to assure safety and to facilitate global supply of high quality vaccines by streamlining the overall batch release process, there is a need to develop and validate new state-of-the-art alternatives methods and to harmonize regulatory requirements. For this purpose, collaborative studies organized by WHO, EDQM, or FDA are critical and should be encouraged. These collaborative studies are focused on either evaluating new reference materials or

exploring/validating alternative methods. They have been extremely useful in introducing alternative tests for in vitro potency assessments of vaccines (IPV, Hepatitis A vaccine) or new methods such MAPREC test (Chumakov et al. 1991) for neurovirulence testing of oral polio vaccines, reducing or eliminating the use of monkeys in this test.

Finally, in addition to these collaborative studies, there is a need for convergence of regulatory requirements to avoid the multiple testing performed to comply with several regulations. For this convergence goal, WHO could play a critical role in order to facilitate the scientific discussion between the stakeholders in public health (Regulatory agencies, manufacturers, international agencies) and to promote the introduction of new methods consistent with 3Rs principles worldwide. Pharmacopeia requirements and guidelines should be developed using these scientific discussions and rationales and without the a priori that compendial methods, implemented several decades ago, are always gold standards. This is probably the only way to promote the introduction of alternatives and modern methods. Finally, in the absence of regulatory requirements for a particular test or product in one country, a mutual recognition of other existing compendial monographs should be encouraged in order to avoid revalidation exercise that will not change the performances of the established compendial test.

16.13 Mutual Recognition Among Regulatory Authorities

As it is the case within Europe and between Europe and many countries, mutual recognition among regulatory agencies in lot release process, particularly lot release testing should be encouraged to avoid repetitive testing and delays in product release. This would also help in avoiding a regulatory dilemma when a lot met the lot release criteria at one regulatory agency and failed to meet such criteria at another regulatory agency. The WHO plays an important role and can play an increasing role in this aspect by accrediting laboratories at regulatory agencies for lot release testing.

References

Akama K, Kameyama S, Otani S, Sadahiro S, Murata R (1971) Reversion of toxicity of diphtheria toxoid. Jpn J Med Sci Biol 24:183–187

Bhandari SK, Gupta RK, Sharma SB, Pandey K, Maheshwari SC, Ahuja S, Saxena SN (1990) Testing of adsorbed diphtheria-pertussis-tetanus (DPT) vaccine for freedom from abnormal toxicity. Vaccine 8:105–106

Burn JH, Finney DJ, Goodwin LG (1950) Biological standardization. Oxford University Press, New York

Chinese Pharmacopoeia (Ch P) (2010) (books only)

Chumakov KM, Powers LB, Noonan KE, Roninson IB, Levenbook IS (1991) Correlation between amount of virus with altered nucleotide sequence and the monkey test for acceptability of oral poliovirus vaccine. Proc Natl Acad Sci USA 88:199–203

European Pharmacopoeia (Ph. Eur.) (2014a) http://online.edqm.eu/EN/entry.htm

European Pharmacopoeia (Ph. Eur.) (2014b) 1. General Notices, Supplement 8.2, p 3897

European Pharmacopoeia (2013a) Chapter 2.6.1, Sterility, 8th edn

European Pharmacopoeia (2013b) Chapter 2.6.7, Mycoplasmas, 8th edn

European Pharmacopoeia (2013c) Chapter 5.2.3, Cell substrates for the production of vaccines for human use, 8th edn

European Pharmacopoeia (2013d) Chapter 2.6.16, Tests for extraneous agents in viral vaccines for human use, 8th edn

European Union (2013) Official control authority batch release. Human vaccine and blood derived medicinal products, EU administrative procedure for official control authority batch release, Aug 2013

EDQM (2013) Official control authority batch release (OCABR) for human biologicals: vaccines, blood and plasma derivatives. http://www.edqm.eu/en/human-biologicals-611.html

European Commission (2001) Volume 4, EU guidelines for good manufacturing practice for medicinal products for human and veterinary use. Annex 16, certification by a qualified person and batch release (under revision). http://ec.europa.eu/health/documents/eudralex/vol-4/index_en.htm

Federal Register (1993) Guidance on alternatives to lot release for licensed biological products, vol 58, no 137, p 38771, 20 July 1993

Federal Register (1995) Interim definition and elimination of lot-by-lot release for well characterized therapeutic recombinant DNA-derived and monoclonal antibody biotechnology products, vol 60, no 236, p 63048, 8 Dec 1995

Federal Register (1998a) Revisions to the general safety requirements for biological products, vol 63, no 75, p 19399, 20 April 1998

Federal Register (1998b) Revisions to the general safety test requirements for biological products. vol 63, no 150, p 41718, 5 Aug 1998

Gupta RK (1996) Is the test for abnormal toxicity, general safety or innocuity necessary for vaccines? Vaccine 14(1718):1996

Gupta RK, Collier RJ, Rappuoli R, Siber GR (1997) Differences in the immunogenicity of native and formalinized cross reacting material (CRM_{197}) of diphtheria toxin in mice and guinea pigs and their implications on the development and control of diphtheria vaccine based on CRMs. Vaccine 15:1341–1343

Gupta RK (2011) Reference standards for vaccines. Presentation made at the IABS conference, reference standards for therapeutic proteins: their relevance, development, qualification and replacement, Bethesda, MD, 20–21 Sept 2011. International Alliance for Biological Standardization. http://www.researchgate.net/profile/Rajesh_Gupta36/publications?pubType=inProceedings

Health Canada (2005) Guidance for sponsors, lot release program for schedule D (biologic) drugs. Published by authority of the Minister of Health, June 2005. http://www.hc-sc.gc.ca/dhp-mps/alt_formats/hpfb-dgpsa/pdf/brgtherap/gui_sponsors-dir_promoteurs_lot_program-eng.pdf

Indian Pharmacopoeia (2014) http://www.indianpharmacopoeia.in/

International Conference on Harmonisation (2005) Validation of analytical procedures: text and methodology, ICH harmonised tripartite guideline, Nov 2005. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf

Japanese Pharmacopoeia (JP) (2011) http://jpdb.nihs.go.jp/jp16e/

Nathanson N, Langmuir AD (1963a) The cutter incident. Poliomyelitis following formaldehyde-inactivated poliovirus vaccination in the United States during the spring of 1955 I. Background. Am J Hyg 78:16–28

Nathanson N, Langmuir AD (1963b) The Cutter incident. Poliomyelitis following formaldehyde-inactivated poliovirus vaccination in the United States during the spring of 1955 II. Relationship of poliomyelitis to Cutter vaccine. Am J Hyg 78:29–60

Schwanig M, Nagel M, Duchow K, Krämer B (1997) Elimination of abnormal toxicity test for sera and certain vaccines in the European Pharmacopoeia. Vaccine 15:1047–1048

Stokes W, McFarland R, Kulpa-Eddy J, Gatewood D, Levis R, Halder M, Pulle G, Kojima H, Casey W, Gaydamaka A, Miller T, Brown K, Lewis C, Chapsal JM, Bruckner L, Gairola S, Kamphuis E, Rupprecht CE, Wunderli P, McElhinney L, De Mattia F, Gamoh K, Hill R, Reed D, Doelling V, Johnson N, Allen D, Rinckel L, Jones B (2012) Report on the international workshop on alternative methods for human and veterinary rabies vaccine testing: state of the science and planning the way forward. Biologicals 40(5):369–381

United States, Food and Drug Administration (2010) Characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines for infectious disease indications

United States, Food and Drug Administration (2013a) Requests for samples and protocols; official release, 21 CFR 610.2

United States, Food and Drug Administration (2013b) General safety, 21 CFR 610.11

United States, Food and Drug Administration (2013c) Sterility, 21 CFR 610.12

United States Pharmacopoeia (USP) (2013) http://www.uspnf.com/uspnf/login

United States Pharmacopoeia (2011) Chapter <71> Sterility tests. Pharmacopeial Forum USP 33-NF 28. Rockville, MD: The United States Pharmacopeial Convention, Inc., 2011

World Health Organization (1990) Requirements for diphtheria, tetanus, pertussis and combined vaccines. In: WHO Expert Committee on Biological Standardization. Fortieth report. Geneva, World Health Organization, Annex 2, WHO technical report series, no. 800

World Health Organization (1992) Guidelines for national authorities on quality assurance for biological products. In: WHO Expert Committee on Biological Standardization. Forty-second report. Geneva, Annex 2, WHO technical report series, no. 822

World Health Organization (1994) Requirements for measles, mumps and rubella vaccines and combined vaccines (Live). In: WHO Expert Committee on Biological Standardization. Forty-third report. Geneva, World Health Organization, Annex 3 WHO technical report series, no. 840

World Health Organization (1999) Recommendations for the production and control of poliomyelitis vaccine (oral). In: WHO Expert Committee on Biological Standardization. Fifty report. Geneva, World Health Organization, Annex 1, WHO technical report series no. 904

World Health Organization (2004) Recommendations for the preparation, characterization and establishment of international and other biological reference standards. In: WHO Expert Committee on Biological Standardization Fifty-fifth report. Geneva, World Health Organization, Annex 2, WHO technical report series, no. 932

World Health Organization (2005) Guidelines to assure the quality, safety and efficacy of live attenuated rotavirus vaccines (oral). In: WHO Expert Committee on Biological Standardization. Fifty-sixth report. Geneva, World Health Organization, Annex 3, WHO technical report series, no. 941

World Health Organization (2011a) Guidelines to assure the quality, safety, and efficacy of recombinant human papillomavirus virus-like particle vaccine. In: WHO Expert Committee on Biological Standardization. Fifty seventh report. Geneva, World Health Organization, Annexure 1, WHO technical report series, no. 962

World Health Organization (2011b) Guidelines for assuring the quality, safety and efficacy of meningococcal A conjugate vaccines. In: WHO Expert Committee on Biological Standardization. Fifty seventh report. Geneva, World Health Organization, Annexure 2, WHO technical report series, no. 962

World Health Organization (2011c) Recommendations for inactivated Japanese encephalitis vaccine for human use. In: WHO Expert Committee on Biological Standardization. Fifty eighth report. Geneva, World Health Organization, Annex 1, WHO technical report series, no. 963

World Health Organization (2013a) Recommendations to assure the quality, safety and efficacy of pneumococcal conjugate vaccines. In: WHO Experts Committee on Biological Standardization. Sixtieth report. Geneva, World Health Organization, Annex 3, WHO technical report series, no. 977

World Health Organization (2013b) Recommendations to assure the quality, safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration. In: WHO Experts Committee on Biological Standardization. Sixtieth report. Geneva, World Health Organization, Annex 4, WHO technical report series, no. 977

World Health Organization (2013c) Guidelines for Independent Lot Release of Vaccines by Regulatory Authorities. In: WHO Experts Committee on Biological Standardization. Sixty first report. Geneva, World Health Organization, Annex 2, WHO technical report series, no. 978

World Health Organization (2013d) Recommendations to assure the quality, safety and efficacy of recombinant hepatitis B vaccines. In: WHO Experts Committee on Biological Standardization. Sixty first report. Geneva, World Health Organization, Annex 4, WHO technical report series, no. 978

World Health Organization (2013e) Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines. In: WHO Experts Committee on Biological Standardization. Sixty first report. Geneva, World Health Organization, Annex 5, WHO technical report series, no. 978

World Health Organization (2013f) Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated) In: WHO Expert Committee on Biological Standardization. Sixty second report. Geneva, World Health Organization, Annex 2, WHO technical report series, no. 979

World Health Organization (2013g) Recommendations to assure the quality, safety and efficacy of BCG vaccines. In: WHO Experts Committee on Biological Standardization. Sixty second report. Geneva, World Health Organization, Annex 3, WHO technical report series, no. 979

World Health Organization (2013h) Recommendations for the evaluation of animal cell cultures as cell substrates for the manufacture of biological medicinal products and for the characterization of cell banks. Sixty first report. Geneva, World Health Organization, Annex 3, WHO technical report series, no. 978

Chapter 17
Dendritic Cell Targeting Vaccines

Yoonkyung Do and Bradford Powell

Abbreviations

AIDS/HIV	Acquired immune deficiency syndrome and human immunodeficiency virus disease
APCs	Antigen presenting cells
BAL	Bronchoalveolar lavage
CD40L	CD40 ligand
CFA	Complete freund's adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
CFU	Colony forming unit
CHO	Chinese hamster ovary
CMI	Cell-mediated immunity
CTL	Cytotoxic T lymphocytes
DCs	Dendritic cells
DCR	DC-receptor
DC-SIGN/CD209	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DEC-205/CD205	Dendritic and epithelial cells 205 kDa
EBNA-1	Nuclear antigen 1 of Epstein-Bar-Virus
EBV	Epstein Bar Virus
EDE	Glu-Asp-Glu
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot assay
F1	Capsular antigen fraction 1
FACS	Fluorescence activated cell sorting

Y. Do (✉)
School of Life Sciences, Ulsan National Institute of Science and Technology, UNIST-gil 50, Ulsan 689-798, Republic of Korea
e-mail: yoonkyungdo@gmail.com

B. Powell (✉)
Cernomics Solutions, P.O. Box 1477, Frederick, MD 21702, USA
e-mail: bpowell@mris.com

B. Powell
PharmAthene, Inc., Annapolis, MD 21401, USA

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6_17

Fcγ	Cell-surface receptors that bind the Fc portion of IgG
GLA	Glucopyranosyl lipid A
GM-CSF	Granular macrophage colony stimulating factor
HER2	Human epidermal growth factor receptor
HPV	Human papilloma virus
ICS	Intracellular cytokine staining
IFN	Interferon
I.M.	Intramascular
I.N.	Intranasal
I.P.	Intraperitoneally
KLH	Keyhole limpet hemocyanin
KO	Knock-out
LcrV	Low-calcium response virulence protein
LD_{50}	Lethal dose, 50 %
LNs	Lymph nodes
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAdCAM	Mucosal addressin cell adhesion molecule
MALP-2	Macrophage-activating lipopeptide-2
MCM	Monocyte-conditioned medium
MDA5	Melanoma differentiation-associated protein 5
MMR	Macrophage mannose receptor
Mo-DCs	Monocyte-derived DCs
MPLA	Monophosphoryl lipid A
NHP	Nonhuman primates
NK	Natural killer
NKT	Natural killer T
NOG	NOD/LtSz-*scid IL2Rγ*^{null}
NOS2	Nitric oxide synthase 2
NYVAC	New York vaccinia virus
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cell
PGE2	Prostaglandin E2
Pgm-	Pigmentation negative strain
PHRI	Public health research institute
Poly IC	Polyinosinic: polycytidylic acid
Poly ICLC	Poly IC with poly-L-lysine and carboxymethyl cellulose
S.C.	Subcutaneously
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STAT	Signal transducer and activator of transcription
T3SS	Type III secretion system
TAPs	Transporters for antigen presentation
TLR	Toll-like receptors
TNF	Tumor necrosis factor

Treg	Regulatory T cell
TT	Tetanus toxoid
Y. pestis	Yersinia pestis
Yops	Y. pestis effector molecules

17.1 Introduction

Vaccines are effective and have long-lived immunity only to the extent that they induce T cell memory directed against appropriate target antigens. T cell memory controls humoral and cell-mediated anamnestic responses, and appropriate targeting insures effectual immune action against the etiological agent of disease. This dual requirement for an efficacious vaccine is achieved by delivering target antigen epitopes which drive successful processing, presentation, and T cell induction. The inclusion of such antigens has been somewhat empirical, though modern biotechnology and recombinant engineering has enabled the rational design of potent new chimeric biomolecules. Several types of vaccines have been described in this book, categorized by antigen source as being natural, modified, engineered, combination, or expressed in vivo; exemplified by whole cell-, live attenuated-, subunit-, conjugate- or DNA vaccines. This chapter describes another category of vaccine differentiated by its strategy of antigen presentation. Dendritic cell vaccines share a targeting strategy in which specific antigen is delivered to dendritic cells (DCs) via antibodies directed against their specific endocytic receptors. DC-targeting vaccines have been under development for nearly a decade and recent advances have greatly improved the DC vaccine strategy. This chapter summarizes the development and use of a recent DC-targeting vaccine platform as tested against disease models of particular importance. We also provide a generalized method for development and application against other antigens.

In spite of past advances in biotechnology and the exponential accumulation of genomic data and biomolecular information, some diseases and pathological conditions are still recalcitrant to effective prophylaxis or therapy. To date, conventional vaccine technologies have been problematic or ineffective against several important infectious diseases (i.e., tuberculosis, malaria, HIV/AIDS) and noninfectious diseases (e.g., cancer). Beginning in 1995 (Hsu et al. 1996; Ridgway 2003), the new vaccine approach of DC-targeting entered clinical testing, and continues to gain interest, particularly in areas of cancer prevention and treatment. Recent progress in the DC-targeting strategy has improved the engineering, manufacture and efficacy of targeting products, and promises to revolutionize vaccination against difficult diseases. The first section describes unique and centrally important features of DCs with regard to vaccine biology, and focuses on conventional features and future challenges of DC-based vaccine development.

17.1.1 Dendritic Cells

We begin with a brief overview of DC immunobiology and description of how DCs are pivotal to this vaccine technology platform. Extensive information on DCs immunobiology is available elsewhere (Banchereau and Steinman 1998; Steinman and Banchereau 2007) to which the reader is guided for detailed and current reviews of background knowledge. Basic research and lessons learned from clinical trials have well established that effective vaccines must induce enhanced cell-mediated immune responses with memory in concert with humoral immunity, and must involve mucosal aspects of protection as related to disease pathogenesis. DCs are very capable of meeting these diverse requirements, as is illustrated throughout this chapter. DCs are specialized antigen presenting cells (APCs) that initiate and control immune responses (Banchereau and Steinman 1998; Steinman and Banchereau 2007). Foremost, DCs are potent T cell stimulators.

Immature DCs, as sentinels have a critical role in establishing adaptive immunity to a newly encountered threat; They are abundant at body surfaces including mucosal areas where most infections occur, and they actively acquire antigens by extending their dendrites through the tight junction interfaces of epithelial cells (Rescigno et al. 2001; Niess et al. 2005). DCs have a specialized endocytic system comprised of various uptake receptors and antigen processing compartments (Trombetta and Mellman 2005), which underlie their central and unique role in innate immunity. After encountering nonself entities, DCs bearing antigen then migrate into local lymphoid organs (Cyster 1999; Randolph et al. 2005), which allows efficient selection of specific clones from the diverse antigen-specific repertoire, often less than one in 10^5 lymphocytes. This facilitates presentation of processed antigens to $CD4^+$ and $CD8^+$ T cells, glycolipids to NKT cells, as well as native antigens to B cells leading to diverse forms of adaptive immunity (Mellman and Steinman 2001). When a pathogen unsuccessfully infects a DC, microbial proteins are imported to the cytoplasm where they are clipped by proteasomes and then transported to the rough endoplasmic reticulum through TAPs (transporters for antigen presentation). Processed peptides of about nine amino acids in length are loaded onto the antigen-binding cleft of newly synthesized MHC class I molecules, and the MHC I-peptide complexes reach the surface during transit through the Golgi apparatus. This classical endogenous pathway of DC processing leads to activation of antigen-specific $CD8^+$ T cells (Pamer and Cresswell 1998). In contrast, the exogenous pathway begins when proteins are delivered to DCs through phagocytosis or receptor-mediated endocytosis. Within non-lysosomal transport vesicles, the processed peptides, either preloaded or newly loaded onto MHC class II, are then presented to specific $CD4^+$ T cells (Turley et al. 2000). Importantly, the vesicles contain not only MHC II-peptide complexes but also CD86, which is a co-stimulatory molecule that enable DCs to stimulate $CD4^+$ T cells very efficiently (Engering and Pieters 2001). Another pathway and special feature of DCs is "cross-presentation," which is essential for induction of enhanced T cell immunity in vaccine biology (Albert et al. 1998; Heath et al. 2004). During cross-presentation,

peptides from extracellular antigens are presented within the MHC class I complex. This cross-presentation capacity allows DCs to elicit $CD8^+$ CTL (cytotoxic T lymphocytes) reaction without infection or endogenous biosynthesis of proteins in contrast to the "classical endogenous pathway." For example, proteins derived from immune complexes arising via the Fcγ receptor (see Abbreviations), or from dying cells via phosphatidylserine receptor, or from microbes inactivated by ultraviolet, heat or chemical treatments, each gain access to the cytoplasm of DCs resulting in protein ubiquitination, proteasomal degradation, and routing via TAP-mediated transportation to the surface. Thus, many non-replicating antigens, including protein subunit vaccines and chemically inactivated vaccines, can "cross" over to MHC class I products of DCs and elicit $CD8^+$ T cell responses. This cross-presentation by DCs is critical to the effectiveness of vaccines intended against chronic intracellular infectious diseases and cancers.

In the steady state, or in the absence of immune system perturbation, DCs remain in an immature status, and can actively silence untoward immune activation against self and environmental proteins (Hawiger et al. 2001). This induction of tolerance by DCs is also important in immune-regulation, and is particularly important in developing treatments for transplantation, allergy, or autoimmunity. Therefore, awareness of DC "maturation" and tolerance is necessary to exploit DCs for vaccine development (Reis e Sousa 2006). A spectrum of stimuli has been identified as DC maturation signals. One category important for signaling in the presence of microbes is the toll-like receptor (TLR) (Akira et al. 2001; Medzhitov 2001), and a second is TNF signaling by other lymphocytes including NK, NKT, T, and B cells (Fujii et al. 2004). Although different stimuli can affect DC functioning at different levels, matured DCs generally: reduce antigen capturing and processing, increase the expression of co-stimulatory molecules (i.e., CD40, CD80, CD86), increase production of various cytokines and chemokines, and increase migration to draining lymph nodes (LNs) and the survival of DCs (Banchereau and Steinman 1998; Steinman and Banchereau 2007). These series of processes convert DCs from functioning as "immune silencer" to "immune stimulator" and, therein, bridge innate, and adaptive immunities. This was the subject of the 2011 Nobel Prize in Physiology or Medicine, co-awarded posthumously to Dr. Ralph Steinman for his important discoveries in this area. Recent insights also suggest that different functions by distinct DC subsets are responsible for such various and almost contradictory immune responses as tolerance versus activation (Pulendran et al. 1999; Shortman and Liu 2002; Shortman and Naik 2007).

Based on the cell physiology discussed above, the advantages of utilizing DCs in vaccine biology can be summarized in two aspects: the quantity and the quality of induced immune responses. For example, DCs prime antigen-specific $CD4^+$ T cells efficiently and differentiate them into IFN-γ secreting Th1 type $CD4^+$ T cells. DC-delivered peptides are observed to induce specific immune responses that are 100–1,000 times more efficiently than nonspecifically delivered peptides mixed in Complete Freund's Adjuvant (CFA) (Hawiger et al. 2001). Th1 helper T cells play an important role in activating macrophages to fight against intracellular bacteria and protozoa. They provide aid to $CD8^+$ T cells in defending against viral infections and

cancers, and help antigen-specific B cells to proliferate and produce antibodies as well. In addition, DCs can directly stimulate $CD8^+$ T cells and B cells. In this circumstance, the functional affinity of the $CD8^+$ T cells is improved and antibody secretion and isotype switching are enhanced, including IgA production which is important in mucosal protection. Moreover, DCs can induce T cell memory for both $CD4^+$ and $CD8^+$ responses (Steinman 2007; Steinman and Banchereau 2007). Thus, it is essential to harness the DC's central and versatile role in controlling immune system function while engineering improvements in vaccine efficacy, potency, and longevity.

17.1.2 Conventional DC-Based Vaccine Approach

Valuable concepts of DC-based vaccine approaches have been successfully demonstrated in numerous experimental animal models. To date, the DC-based vaccine platform has proven to be largely safe and able to induce antigen-specific T cell responses in patients. As discussed further below, Table 17.2 lists clinical vaccine trials using a DC strategy, all but one of which employs the conventional method. The difference between conventional and new DC-based vaccine strategies is described in Sect. 17.1.3 below and illustrated in Fig. 17.1. Here, we first describe

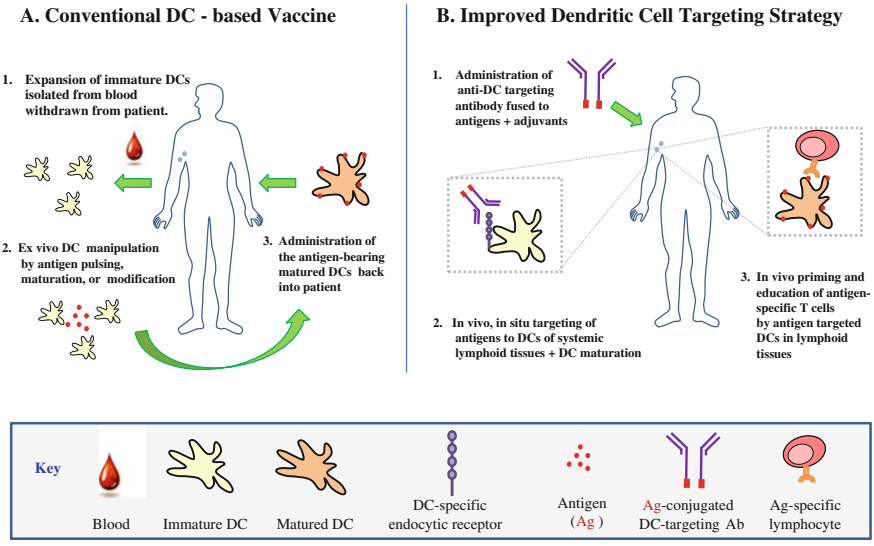


Fig. 17.1 Comparison of conventional and new DC-targeting vaccine strategies. The principal stages and components of dendritic cell-based vaccine strategies are depicted for the conventional (**a**) and new, improved (**b**) approaches. Diagrammatic representations of critical components are given in the key below for blood, immature DC, matured DC, DC-endocytic receptor, antigen (*red*), antigen-conjugated DC-targeting antibody, and antigen-specific lymphocyte. *Arrows* depict stages that involve medical or laboratory handling of material that is withdrawn from or administered to the patient. Numbered stages occur either ex vivo or in vivo as described

the general procedure of conventional DC-based vaccines used in clinical applications with some discussion methods used in isolating DC precursors, antigen pulsing, and ex vivo manipulations, as well as suggested improvements to be considered for future DC-based vaccine approach.

Two general sources of DC precursors have been facilitated for use in the conventional DC strategy, $CD34^+$ proliferating hematopoietic stem cells, and $CD14^+$ monocytes (Banchereau et al. 2001; Steinman and Dhodapkar 2001). Monocyte-derived DCs (Mo-DCs) have been extensively used in clinical applications, partly because they are easier to be obtained and a more homogenous population than $CD34^+$-derived DCs. The methods for isolating DC precursors and their subsequent culturing have improved so that large numbers of homogenous Mo-DCs (i.e., 300–500 million mature DCs per apheresis) can be generated with GM-CSF (granularmacrophage colonystimulating factor) and IL-4 to give a product of consistent quality (Berger et al. 2002). While increased yields of homogenous DCs would be ideal for use in clinical trials, better understanding is needed with regard to the specialized immune functions induced by distinct DC subsets.

For introducing or "pulsing" antigens into DCs, various forms of antigen and delivery vehicle have been applied, including transfection of RNAs or immune-dominant peptides, and antibody-coated tumor cells, whole cell lysates, or necrotic cells (Schuler et al. 2003). Using cells or cell lysates does not require identification of antigenic epitopes, so is presumed to save time and effort compared to using immune-dominant peptides or RNAs. However, the latter approaches can be used in patients regardless of tumor type and does not require a preparation of large amount of antigen. Moreover, it might provide DCs with selective antigens leading to enhanced antigen-specific immune responses.

As described in Sect. 17.1.1, activating DCs into "mature" status is critical for immune stimulation. There are several established "maturation cocktails" including TLR ligands (e.g., MPLA, poly IC) or inflammatory cytokines (e.g., TNF-α). Monocyte-conditioned medium (MCM) composed of IL-1β, TNF-α, IL-6, PGE2, or CD40L represents a standard for Mo-DCs stimulation (Steinman and Pope 2002; Schuler et al. 2003), although a validated clinical standard has not been published. Previous studies reported that Mo-DCs became more homogenous in population diversity after MCM treatment, and they were highly viable and migrated well upon chemotactic signals (Schuler et al. 2003). A critical question remains, however, of how to determine whether the ex vivo-manipulated DCs are truly matured functional and in vivo. So far, various protocols to check either up-regulation of surface co-stimulatory molecules (e.g., CD80, CD86, CD83), cytokine production (e.g., IL-12), or in vitro immune responses using a positive control antigen pulsed together with real pathogenic antigens such as tetanus toxoid (TT) or keyhole limpet hemocyanin (KLH), have been applied. For clinical DC-based vaccines, standardized protocols are needed that provide improved functional aspects of DCs.

Although optimized vaccination schedule, dosage, and routes of DC-based vaccine approaches have yet to be determined, previous trials have demonstrated that tumor-specific T cells could be induced by either intradermal (Fong et al. 2001a), subcutaneous (Schuler-Thurner et al. 2002), intranodal (Jonuleit et al. 2001),

or intravenous (Fong et al. 2001b) injection of ex vivo-manipulated DCs in patients. Given the facts that efficient migration to lymph nodes of the administered DCs is essential for the induction of antigen-specific T cell immunity, and less than 6 % of the Mo-DCs could reach the nodes (Schuler et al. 2003), it can be surmised that improved delivery should be pursued. Although methods are conceived to overcome such migratory limits, including a direct intranodal injection, manipulation of CCL19/CCR7 interaction, or an increased cell count and schedule frequency of DC administration, they bring additional difficulties which have hampered clinical application depending on cancer types. For example, diminished DC functions due to over-manipulation, or induction of unwanted counter immune responses due to already sensitized immune responses have been observed. Therefore, for improved DC-based vaccine approaches, developers must consider such a way to deliver antigen to as many DCs as possible so that effective B and T cell immunity can be induced in systemic lymphoid tissues including mucosal nodes.

Beginning with the first clinical DC vaccination study in 1995 (Hsu et al. 1996), numerous trials have been launched against various cancer diseases and infectious diseases. To date, there are 220 clinical trials worldwide listed that use some form of DC vaccine intervention, as publically listed by www.clinicaltrials.gov (Table 17.2). The majority of these are centered in the US and Canada, followed by Europe and the Asia/Pacifica region. Most studies are in Phase 1 and/or Phase 2, with 33 listed as Phase 3 at the time of this writing. Nearly, all studies target cancer and neoplasms, though 10 target an infectious disease, all of these being against HIV/AIDS. One study uses the new DC-targeting platform described in this chapter, employing a target antigen-fused anti-DC receptor antibody, called the DEC-205-NY-ESO-1 fusion protein vaccine (Study NCT01522820; Table 17.2, italics). While some studies have generated results, these data are not publically accessible.

Although we still need more data to draw broad conclusions on the efficacy of DC-based vaccine approaches, it is clear that valuable proofs of concept in clinical applications have been collected, and the strategy has demonstrated sufficient potential to warrant further pursuit. The new DC-targeting platform is discussed below.

17.1.3 New DC-Targeting Vaccine Approach

The concept of a novel DC-targeting strategy is depicted in Fig. 17.1, where differentiating principles and improvements are compared to conventional DC-based vaccine approaches. The principal advantages of the new targeting strategy is greater targeting efficiency and greater procedural safety through less handling of material introduced into the patient. The strategy is elegant: antigen is couriered directly via an anti-DC receptor antibody. In brief, the new DC-targeting strategy uses a chimeric conjugated antibody in which heavy chain is fused to antigen by recombinant engineering with a systemic DC maturation stimulus (Trumpfheller et al. 2012).

The conjugated antibody targets DCs at various DC-specific endocytic receptors, and the targeted antigens are endocytosed, processed, and presented to antigen-specific T cells by mature DCs. Thus, the new DC-targeting strategy employs natural processes occurring in vivo to achieve antigen loading, DC maturation, enrichment, and presentation. This new strategy circumvents several problems associated with the conventional approach as listed below (see boxed insert). Although both approaches involve a single parenteral product, the conventional approaches require collection of DCs from patient for manipulation and purification before re-introduction, creating more risk to negatively impact quality, safety, and efficacy. Complexities associated with the isolation and culture of DCs, their ex vivo manipulation, and their homing to LNs on reintroduction render the conventional approach generally intractable for analytical measurement and therefore problematic in monitoring for quality control.

Problems with Conventional DC-Based Vaccine Approaches

- Difficulty in isolation and expansion of DCs with proper number and quality
- Inefficacy of the ex vivo cultured DCs in homing to draining LNs and purity
- Potential problems during ex vivo DC manipulation
- Limitations of antigen preparation
- Potential risk to quality (e.g., freezing and thawing cells) and safety due to ex vivo handling and manipulation of material introduced back into the patient

The key of DC-targeting strategy for vaccine biology is harnessing the capacity of DCs as APCs and thus, to maximize a role of DCs immune system, or nature's adjuvant. In other words: (1) by targeting antigens to endocytic receptors, antigen capture and process is significantly enhanced; (2) by administrating DCs stimulus simultaneously, antigen-bearing DCs become true immune stimulators; and (3) by delivering antigens directly to DCs in vivo in situ, critical challenges on DC isolation, ex vivo-manipulation, or migration to the nodes are overcome.

Due to these influential capabilities, the DC-targeting strategy was developed incrementally and extensively wherein several aspects were considered carefully including, the screening and characterization of DC-specific endocytic receptors, generation of monoclonal antibodies with quality control, selection of antigens of interest and disease models, and efficient immune monitoring system to examine the efficacy of the strategy as therapeutic as well as preventive vaccines. Technology discovery and development information on each of these topics is discussed further in following sections of this chapter. We begin with the rationale to select the DEC-205 endocytic receptor for the DC-targeting strategy and lessons learned from first demonstration using a model ovalbulin antigen (OVA) system (Sect. 17.2). The DC-targeting strategy has been refined using pathogenic disease models, giving

expected results, and some surprise observations (Sects. 17.3 and 17.4). Lastly, various efforts for clinical applications (Sect. 17.4) and future directions (Sect. 17.5) are considered and discussed.

17.2 DC-Targeting Strategy: Technology Discovery with OVA Model Antigen

This section describes the rationale for selecting DEC-205 over other DC-specific endocytic receptor as a targeting molecule, and valuable information on the DEC-targeting strategy as potential prophylactic and preventive vaccine approach. Much of this information was gained from early experiments using the ovalbumin model antigen and OVA transgenic mice system.

17.2.1 The DC Receptor for Endocytosis, DEC-205

Dendritic cells express various receptors for absorptive uptake of antigens, and DC subtypes are classified in part by the identity of these antigen uptake receptors. For example, Fcγ receptors (Regnault et al. 1999), and macrophage mannose receptor (MMR) (Sallusto et al. 1995) are shared with other type of cells, while CD205 (DEC-205) (Jiang et al. 1995), CD207 (Langerin) (Valladeau et al. 2000), CD209 (DC-SIGN) (Engering et al. 2002) or BDCA-2 (Dzionek et al. 2001), are more DC-restricted receptors. Among these, DEC-205 has received intense interest for use in the DC-targeting strategy due to the following features. First, this endocytic receptor mediates the efficient processing and presentation of antigens on MHC class I and II products in vivo (Jiang et al. 1995). An early study demonstrated that the DEC-205 receptor targets late endosomes or lysosomes rich in MHC class II products, while the homologous MMR was found in more peripheral endosomes (Mahnke et al. 2000). In addition, DEC-205 showed enhanced surface binding, up-taking, and presentation in comparison of MMR. These properties of DEC-205 were mediated by a membrane-proximal region with a coated pit sequence for up-taking, and a distal region with an EDE triad (Glu-Asp-Glu) for the unusual deeper targeting (Mahnke et al. 2000). Second, DEC-205 is expressed at high levels on lymphoid tissue DCs, and particularly in the T cell areas of lymphoid organs, which might enhance antigen-specific DC/T cell contact (Witmer-Pack et al. 1995). Moreover, monoclonal antibody against DEC-205 (anti- or αDEC-205 mAb) demonstrates a target selectively for DEC-205$^+$ DCs in mice (Hawiger et al. 2001). Based on these properties, any protein joined to the DEC-205$^+$ DCs via αDEC-205 mAb was anticipated to be processed and presented efficiently (now, we define this as DEC-targeting). This capability was demonstrated with greatly enhanced induction of antigen-specific CD4$^+$/CD8$^+$ T cell responses (Hawiger et al. 2001).

Given the fact that less than one in 10,000 naïve T cells are able to respond to a specific antigen in general, its stimulation is difficult to detect by conventional immune assays. To aid development and testing, ovalbumin (OVA) was selected as a model antigen within OVA transgenic mice, such as OT-I (transgenic T cell receptor for MHC class I-restricted OVA peptide, amino acids 257–264, SIINFEKL) or OT-II (transgenic T cell receptor for MHC class II-restricted OVA peptide, amino acids 323–339, ISQAVHAAHAEINEAGR). This experimental system proved to be very useful in the analysis of antigen-specific T cell immunity in early exploration of the DC-targeting strategy. When graded doses of the anti-DEC-205 antibody conjugated to OVA protein (αDEC:OVA) were subcutaneously (s.c.) injected into the mice, OVA antigen was efficiently presented to OVA-specific $CD8^+$/$CD4^+$ T cells in vitro, as well as in vivo by $CD11c^+$ DCs, but not by $CD11c^-$ DCs or B cells in the draining lymph nodes. These findings indicated high specificity of the DEC-targeting strategy (Bonifaz et al. 2002). In case of MHC class I presentation by $CD11c^+$ DC after αDEC:OVA targeting, transporter associated with antigen processing (TAP) was required which suggested an exogenous MHC class I pathway (Jung et al. 2002). Interestingly, both efficient targeting and peripheral $CD8^+$ T cell tolerance via presentation on MHC class I were observed under steady state or in the absence of infection (Bonifaz et al. 2002). Briefly, in the absence DC stimuli, as achieved experimentally by omitting anti-CD40 mAb (αCD40), OVA-specific $CD8^+$ T cells first proliferated then died by 12–14 days. T cells were not found in lymph nodes, spleen, bloods, or peripheral tissues such as lung. Moreover, there were no recall T cell responses to rechallenge with OVA plus a strong adjuvant such as CFA, which demonstrated the deletion of the T cells or the induction of peripheral tolerance by DEC-205 receptor-mediated antigen delivery. In contrast, when αDEC:OVA was administered in the presence of DC maturation, such as injected αCD40, OVA-specific $CD8^+$ T cells proliferated, produced IL-2 and IFN-γ, and strongly responded to the rechallenge. This demonstrated two important points: first, as previously known, a "danger signal" is required to induce immune stimulatory function of DCs (Bendelac and Medzhitov 2002; Steinman and Banchereau 2007) (see Sect. 17.1.1 for more information); and second, that DEC-205 provides efficient receptor-mediated antigen processing and presentation for MHC class I in vivo, leading to peripheral tolerance with small amounts (see Sect. 17.2.2 for more information). This latter effect typically requires repeated injections of large amounts of proteins or peptides. DEC-205 might play an efficient role for developing tolerance to harmless environmental proteins and self-proteins on MHC class I by continuously deleting those T cells in vivo. Since injection of only αDEC:OVA neither increased DC numbers nor induced DC maturation, these data proved that a coupled target antigen was required in this DC-targeting strategy (Bonifaz et al. 2002).

Based on these findings, the DC-targeting strategy was proposed as an improved DC-based vaccine approach. The effectiveness and practical utility of this platform rests on unique composition of the active pharmaceutical ingredient, comprising αDEC-205 conjugated to target antigen, and adjuvant, comprising αCD40 to induce

strong antigen-specific $CD4^+/CD8^+$ T cell immune responses. Demonstrations of this new DEC-targeting strategy in model diseases are described in Sects. 17.3 and 17.4 of this chapter.

17.2.2 Highly Efficient CD4/CD8 T-Cell Immunity

The immunostimulatory efficiency of DC-targeting was found to be very efficient compared to the standard subunit vaccine approach using purified antigen. For example, more than 1,000-fold increased efficiency was observed for the OVA antigen. In escalating dose-response tests of αDEC:OVA with αCD40 (fixed at 25 μg/mouse) injected into the mice, and OVA-specific $CD4^+/CD8^+$ T cells were assessed by proliferation and by ELISPOT detection of IFN-γ secretion (Bonifaz et al. 2004). A minimum of 2,500 ng non-targeted soluble OVA was required to elicit OVA-specific $CD8^+$ T cell proliferation, while just 2 ng of the DEC-targeted OVA gave the same response. Thus, comparing DEC-targeting delivery to a non-targeted soluble protein administration 1,000 times and 50 times less amount of antigen, respectively, were required to achieve similar level of OVA-specific $CD8^+$ and $CD4^+$ T cell induction. Beyond efficient T cell proliferation and IFN-γ production, DEC-targeting was also observed to induce functional cytotoxic T lymphocytes (CTLs) (Bonifaz et al. 2004).

Interestingly, the efficacy of DEC-targeting for induction of antigen-specific T cell immune responses was more prominent than other vaccination methods tested. For example, mice were injected s.c. with either: (1) splenic DCs pulsed ex vivo with 10 μg/ml of αDEC:OVA and αCD40, (2) 500 μg of the non-targeted soluble OVA protein in CFA, (3) 50 μg of the non-targeted soluble OVA protein with αCD40, (4) 50 μg of OVA peptide (SIINFEKL) with αCD40, or (5) 50 ng of OVA containing αDEC:OVA with αCD40. Following 7 and 30 days after vaccination, OVA-specific T cell expansion was measured by double staining with CD62L and K^b-SIINFEKL-PE tetramer from lymph nodes and spleens. The data showed that at both days, DEC-targeting was the most effective among the group in the induction of the antigen-specific T cell immune responses. The frequency of OVA-specific $CD8^+$ T cells was ∼5 % with 50 ng of the OVA containing αDEC:OVA plus αCD40 treatment at day 7 in spleen, while those of the 50 μg soluble OVA protein (1,000 × molar excess of protein) plus αCD40 group and the 50 μg OVA peptide (467 molar excess of processed peptide) plus αCD40 group were around 1 and 0.2 %, respectively. The frequency of the expanded T cells secreting IFN-γ was the highest in the DEC-targeting group as well. Even after 30 days, about 1.3 % of the OVA-specific $CD8^+$ T cells were detected in the group immunized with the DEC-targeting, and such was not detectable with other vaccination strategies demonstrating the efficacy of the DEC-targeting strategy in the induction of T cell immunity (Bonifaz et al. 2004).

17.2.3 Systemic Antigen Presentation and Durability

It was surmised that the DEC-targeting strategy offered an opportunity for improved efficient systemic antigen delivery given the fact that few ex vivo-manipulated DCs were found in the draining lymph nodes of reinjected patients using the conventional DC-based vaccine approach.

In order to test the rate and the durability of the DEC-targeting antibody in lymphoid tissues, $Alexa_{488}$-conjugated DEC-targeting antibody was injected s.c. into the mice and tracked at various time points (Bonifaz et al. 2004). As early as 30 min after vaccination, the DEC-targeting antibody was bound not only to the $CD11c^+$ DCs in draining lymph nodes but also to the spleen DCs. At 6 h post injection, ~50 % of the DCs in the draining lymph nodes and 40 % of the DCs in distal lymph nodes were targeted, and as late as 3 days after injection, the DEC-targeting antibody was detected in all sites tested. Considering at least 6 h in transit for carriage of the antigen by peripheral DCs to the draining lymph nodes, and that they would not easily move to other organs (Kamath et al. 2000, 2002) (i.e., distal lymph nodes and spleens), this study demonstrated that the DEC-targeting strategy greatly enhanced systemic antigen delivery to the DCs in vivo.

Unexpectedly, the antigen products delivered by the DEC-targeting antibody, particularly those processed on MHC class I products, persisted for longer periods than the typical half life of many DCs (i.e., less than 2 days) (Kamath et al. 2002; Bonifaz et al. 2004). When OVA antigens were given to the mice in two forms-either targeted via αDEC:OVA or as non-targeted soluble OVA-at different time points (1, 3, 7, 15 days) before transferring the OVA-specific T cells, prolonged presentation was detected in the lymph nodes of the DEC-targeted group even after 15 days. In contrast, minimal presentation was detected in the soluble OVA treated group after 7 days. Although further studies are needed to better understand the destiny and mechanisms of antigen targeting, it appears that the DEC-205 receptor is specialized in its capacity for both processing and presentation of antigen as MHC class I products.

Importantly, DC targeted antigens were able to induce stronger antigen-specific T cell responses with durability as well (Bonifaz et al. 2004). For example, IFN-γ secreting $CD8^+$ T cells were examined 14, 21, 60, or 90 days after vaccinating mice with αDEC:OVA, containing 50 ng OVA protein, plus αCD40. Even after 90 days of a subcutaneous single dose of the DEC-targeting antibody vaccination, strong IFN-γ$CD8^+$ T cell responses were detected and efficient CTL activity was still observed. This indicated great efficacy in the formation of effector memory T cells by DEC-targeting.

Taken together, these findings demonstrated several practical attributes of the DEC-targeting strategy as a novel DC-based vaccine platform: (1) fast systemic delivery of antigens to large numbers of DCs in lymphoid tissues with durability; (2) induction of higher frequency of antigen-specific T cell immunity with significantly less antigen; and (3) generation of durable effector memory T cells. These salient features of the DEC-targeting strategy were further demonstrated using a tumor model as shown in the below section.

17.2.4 Vaccine Efficacy

To study the efficacy of the DEC-targeting strategy as a novel DC-based vaccine, mice were injected s.c. with αDEC:OVA or isotype control in the presence and absence of αCD40. Sixty days later, they were challenged with B16 melanoma cells bearing OVA (Bonifaz et al. 2004). Only mice vaccinated with αDEC:OVA plus αCD40 were protected and the protection was mediated primarily by OVA-specific $CD8^+$ T cells with protection by $CD4^+$ T cells to a lesser extent. Testing a therapeutic approach, mice were inoculated with the tumor first (5–10 mm diameter) and subsequently received various forms of OVA antigens, including αDEC:OVA + αCD40, soluble OVA in CFA, ex vivo OVA-pulsed spleen DCs, or PBS/adjuvant alone. The only group to show a therapeutic effect was that treated with the DEC-targeting antibody plus adjuvant. Based on the data showing that primed effector T cells induced by the DEC-targeting strategy emigrated to the lung (Bonifaz et al. 2002), mucosal resistance after a single dose of s.c. vaccination was also tested. Mice were injected either with αDEC:OVA or isotype control plus αCD40, and challenged with intranasal recombinant Vaccinia virus expressing OVA. As evaluated by lung viral titers and weight loss, only αDEC:OVA + αCD40 induced systemic protective immunity including at mucosal surfaces.

In summary, all the data obtained in initial studies with OVA model antigen provided promising aspects of the DEC-targeting strategy as a novel DC-based vaccine approach. In particular, greatly enhanced antigen-specific $CD8^+$ T cell immunity induced by the DEC-targeting strategy drew more attention to its potential application as an efficient vaccine against diseases that require strong cytotoxic killer T cell-mediated immunity such as cancer or viral diseases. To our surprise, the DEC-targeting strategy was more efficient in the induction of antigen-specific responses by $CD4^+$ T cells rather than $CD8^+$ T cells when conjugated with various real pathogen antigens as compared to OVA model antigen. These unexpected results will be explained in detail with a case of plague vaccine development in Sect. 17.3 and with other cases in Sect. 17.4. The ability for cross-presentation by the DEC-targeting strategy will be discussed further in Sect. 17.4 as well.

17.3 DC-Targeting Strategy with a Known Pathogenic Antigen, *Yersinia Pestis* LcrV

Based on results obtained with the OVA model, our DC-targeting strategy was then investigated in various disease models using real disease-related antigens as an improved DC-based vaccine platform. Infectious disease models in which a beneficial role of cell-mediated immunity (CMI) could be investigated were considered first. Plague, caused by *Y. pestis*, was chosen for study among other bacterial infectious disease models due to the ongoing inquiry about host immune protection and the importance of plague as a potential biological weapon or bioterrorism threat.

17.3.1 Breadth and Efficiency of T Cell Immunity Induced by DC-Targeting Strategy

Yersinia pestis (*Y. pestis*) is a Gram-negative bacterium and a etiologic agent of plague or Black Death (Perry and Fetherston 1997) (see also, Chap. 7). Three clinical forms of plague occur in humans depending on the route of bacterial infection. Bubonic plague results from subcutaneous deposition of *Y. pestis* bacillus via infected flea bite. If untreated, bloodstream contamination may result in septicemic plague or pneumonic plague upon infection of the lungs (Perry and Fetherston 1997). Pneumonic plague produces rapid death and natural disbursement through contaminated respiratory droplets. Aerosolized *Y. pestis* may also be created unnaturally as a bioweapon, and is ranked in the highest category of potential biological threat agents due to its relative ease of dissemination and deadly contagiousness. The development of an effective pneumonic plague vaccine is important for military preparedness and homeland security (Inglesby et al. 2000).

There have been numerous efforts to develop human plague vaccines. The only US-licensed plague vaccine (Plague Vaccine USP), a formalin-killed whole cell preparation, was reactogenic, ineffective against pneumonic plague, and ended production in 1999. Live-attenuated plague vaccines such as EV76 are also ineffective against pneumonic plague (Meyer 1970). During pathogenesis, *Y. pestis* employs a macromolecular needle assembly, called the type III secretion system (T3SS), through which toxic effector proteins are injected into the cytoplasm of host cells. These effector proteins disrupt cell signaling pathways critical for phagocytosis, cytokine release, and normal immune-regulation, resulting in paralysis or induction of apoptosis (Ramamurthi and Schneewind 2002). Macrophages and dendritic cells are early target cells during infection (Marketon et al. 2005). Among numerous molecules explored as protective antigens for vaccine development, the low-calcium response virulence protein, LcrV (or V) and the capsular antigen fraction 1, Caf1 (or F1), have received much focus due largely to their immunogenicity. F1 capsule exerts anti-phagocytic activity on macrophages (Du et al. 2002). As a purified subunit antigen, F1 elicits strong antibody responses in animals and humans (Meyer et al. 1974; Andrews et al. 1996). F1-directed humoral immunity is protective against bubonic plague in animal models caused by *Y. pestis* strains expressing F1, but ineffective against engineered F1-negative strains or pneumonic plague models (Meyer et al. 1974; Heath et al. 1998). LcrV is multifunctional, comprising the structural tip of the T3SS injectosome (Brubaker 1991; Ramamurthi and Schneewind 2002), regulating the genetic expression and translocation of other *Y. pestis* effector molecules (Yops) into host cells upon contact (Brubaker 1991), and exerting immunosuppression, including the inhibition of phagocytosis, up-regulation of anti-inflammatory IL-10 (Sing et al. 2002), and down regulation of TNF-gamma (Schmidt et al. 1999). While LcrV antigen alone elicits protective immunity in animal models against infection by either F1-positive or F1-negative strains, combination of LcrV with the F1 antigen provides better protection than either subunit vaccine alone, and protects against pneumonic plague

(Andrews et al. 1996; Powell et al. 2005). Many studies with subunit vaccines composed of F1 and LcrV proteins bound to alhydrogel adjuvant showed protection in small animal models of pneumonic plague and indicated that efficacy results mainly from antibody responses (Williamson et al. 1995; Anderson et al. 1996; Heath et al. 1998; Powell et al. 2005). However, aerosol infection studies with nonhuman primates (NHP) have shown inconsistent protection by the F1/V vaccines. While full protective immunity has been achieved for the cynomolgus monkey (*Macaca fascicularis*), F1/V vaccines have so far failed to elicit demonstrable protection in the African green monkey (*Chlorocebus aethiops*) (Davis et al. 1996; Overheim et al. 2012a, b; Pitt 2004).

Based on this discrepancy in NHP protection, we proposed that the induction of cellular immunity, and the recruitment of DCs in particular, might also be important for an improved pneumonic plague vaccine (Do et al. 2008, 2010) Our proposal was based on the following reasoning. Helper T cells are known to contribute to antibody-based immunity. The $CD4^+$ T cells can secrete high levels of Th1 cytokines (e.g., IFN-γ) that activate macrophages to kill intracellular pathogens, and *Y. pestis* is a facultative intracellular pathogen. In addition, $CD4^+$ T cells are cytolytic on cells bearing MHC class II-targets (Bevan 2004). This helps to explain why mice treated with only exogenous IFN-γ and TNF-α are protected against intravenous challenge by $LcrV^+$ *Y. pestis* (KIM strain, ten median lethal doses), which inhibits *Y. pestis* growth in vivo (Nakajima and Brubaker 1993). Likewise, IFN-γ induces higher antigen-specific systemic immune responses when co-encapsulated with LcrV antigen (Griffin et al. 1998). Moreover, Stat-4 deficient mice, which produce low levels of IFN-γ, are poorly protected from *Y. pestis* strain GB despite producing levels of serum antibody as high as wild type controls (Elvin and Williamson 2004). Previous studies by Smiley and colleagues demonstrated that adoptively transferred *Y. pestis*-primed T cells could protect naïve B cell-deficient mice ($\mu MT^{-/-}$) against intranasal challenge of attenuated *Y. pestis* strain KIM D27 (Parent et al. 2005), and T cells, IFN-γ, TNF-α, and NOS (nitric oxide synthase (2) play important roles in such protection (Parent et al. 2005, 2006). In a subsequent study, they demonstrate that the TNF-α and IFN-γ cytokines contribute to protection against pneumonic plague mediated by anti-F1 and anti-LcrV IgGs in the mouse model (Lin et al. 2010). These data demonstrated a role for Th1 type cellular immunity for an effective immune protection against plague. An additional role for Th17 in protection against pulmonary infection was suggested using B cell-deficient mice primed and boosted with a live attenuated *Y. pestis* strain D27-pLpxL (Lin et al. 2011a). Finally, a dominant CD8 T cell epitope in another *Yesinia* virulence protein, YopE, was recently found to confer protection against pneumonic plague (Lin et al. 2011b). Taken together, an effective plague vaccine may need to prime not only humoral immunity but also strong Th1 or Th1/Th17 type cellular immunity. However, the F1/V subunit vaccine in the presence of alhydrogel is known to induce poor cellular immunity.

Although the above-mentioned studies demonstrate participation by Th1 type cellular immunity in protecting mice against plague, these approaches have limitations for clinical application, the foremost concern being extrapolation from the

mouse model to human disease. Mouse immunology is not wholly predictive of human immune protection (Mestas and Hughes 2004), and pulmonary anatomical differences confound direct comparisons for pathogenesis and treatment of pneumonic diseases (Bates and Irvin 2003). Another concern is the pathogen model and whether the strain and route of infection represent the actual disease condition intended for prophylaxis. For example, *Y. pestis*-specific T cells from mice are derived from in vivo/in vitro stimulation with attenuated whole organism, such as *pgm-* (pigmentation negative strain), which have been already reported to be inefficient against pneumonic plague in humans (Meyer 1970). Mouse challenge experiments performed using an attenuated *Y. pestis* strains in place of a virulent strain, such as CO92, potentially misrepresent the efficacy of the vaccine regimen. Therefore, it is a necessary to design a vaccine that could induce strong T cell immunity with clinical relevance and confer protection against pneumonic plague by a virulent strain.

In order to induce broad and efficient T cell immunity against *Y. pestis*, we developed a DEC-targeting strategy that delivered LcrV protein directly DCs in situ. Full length LcrV sequence was first codon-optimized to improve expression in mammalian transfection system and then cloned in-frame into the heavy chain of anti-mouse DEC-205 mAb (αDEC:LcrV). When mice were immunized subcutaneously with a single dose of this targeted LcrV protein in the presence of poly IC and anti-CD40 (αCD40) as adjuvants, we observed LcrV-specific $CD4^+$ T cell immunity measured by intracellular cytokine staining (ICS) for IFN-γ in three different mouse strains or C57BL/6 ($H\text{-}2^b$), BALB/c ($H\text{-}2^d$), and C3H/HeJ ($H\text{-}2^k$) (Do et al. 2008). The breadth of T cell immunity induced by the DEC-targeting strategy was demonstrated by observation of two (C57BL/6 and C3H/HeJ) and three (BALB/c) distinct $CD4^+$ T cell mimetopes in each haplotype. Induction of broad T cell immunity is critical in considering known human HLA polymorphism as well as immune evasion exploited by the *Yersinia* bacteria. We also observed similar breadth of T cell immunity via an intranasal (i.n.) administration of the DEC-targeted LcrV at mucosal surfaces indicating efficient DC-targeting strategy regardless of routes and target organs (Do et al. 2012) (see Sect. 17.3.4 for more information). The high efficacy of DEC-targeting strategy in the induction of T cell immunity is further supported by the fact that a single and far smaller relative dose of administration is sufficient to induce efficient T cell immunity (see Sect. 17.3.2 for more information). This contrasts with standard protein immunization which requires high concentrations of proteins or peptides, often requiring repeated injections based on strong standard CFA adjuvant to induce detectable $CD4^+$ T cell responses. Furthermore, the DEC-targeted protein generates multifunctional T cells secreting different cytokines such as IFN-γ, TNF-α, or IL-2 at the same time with a higher frequencies when compared to a non-targeted standard protein vaccine suggesting the quality of T cells induced by DC-targeting strategy (Flynn et al. 2011). We observed that LcrV-specific T cells primed by DEC-targeted protein produced IFN-γ, TNF-α, or IL-2, and it will be interesting to confirm whether these are also multifunctional T cells as found in previous studies.

17.3.2 Efficacy: Targeting Versus Non-Targeting

Lowering the effective dosage of a vaccine is an important issue for global health, including in preparation against a bio-terrorism or bio-warfare threat. In comparison to non-targeted soluble protein vaccine, DC-targeting strategy increases the efficiency of inducing LcrV-specific $CD4^+$ T cell responses greatly. For example, 0.1 μg of the conjugated antibody (αDEC:LcrV) per mouse induced an IFN-γ^+CD4^+ T cell response comprising ~0.2 % LcrV-specificity, while non-targeted soluble LcrV protein required at least 10 μg/mouse to induce detectable antigen-specific T cells responses by ICS (Do et al. 2008). Considering the actual LcrV protein amount between these two, in that the conjugated antibody is about one-third of LcrV protein (i.e., 0.1 μg αDEC:LcrV contains 0.03 μg of LcrV), the DC-targeting platform increased the efficiency of antigen-specific T cell induction more than 300-fold. Interestingly, even in using different adjuvants, DC-targeting strategy showed improved the efficiency of T cell induction. Thus, the frequency of LcrV-specific IFN-γ secreting $CD4^+$ T cells induced by 100 μg/mouse of the non-targeted soluble LcrV protein with a standard CFA adjuvant was less than the half of that induced by 10 μg of the DEC-targeted LcrV protein in the presence of poly IC and αCD40 as adjuvants (Do et al. 2008). In general, a range of 1–5 μg of the conjugated DC targeted antibodies per mouse has been used with success in various disease models. When we tested LcrV peptide doses during a re-stimulation assay, we observed that most T cells responded to antigen at 0.2 μM peptide or higher, which also indicated efficient priming in vivo via a targeting strategy. Not only a parenteral but also an intranasal administration of the DC-targeting strategy increased the efficiency of the T cell immunity when compared to non-targeted soluble protein. Previously, we showed that at least 10 × more non-targeted soluble LcrV is required to achieve a similar pulmonary T cell response compared to the DEC-targeting antibody following a prime and boost administered intranasally (Do et al. 2012). Moreover, while the number of IL-2^+ or TNF-α^+ pulmonary $CD4^+$ T cells reaches a plateau when mice are immunized with 3 and 30 μg of non-targeted soluble LcrV, this number significantly increases in mice immunized with 0.3 and 3 μg of the DEC-targeted LcrV, demonstrating again the value of antigen targeting for increasing the efficiency of inducing LcrV-specific cellular immunity (Do et al. 2012). In addition to quantity comparisons, future research should examine the improved quality of T cells induced by DC-targeting strategy, including frequency and features of LcrV-specific memory T cells.

17.3.3 DC Subsets Targeting and B Cell Immunity

The significance of antibody responses in protection against plague is well established (Williamson et al. 1995). Regarding humoral immunity induced by DC-targeting strategy, a previous study showed that 10 μg of the DEC-targeted

LcrV protein with poly IC plus αCD40 as adjuvants induced antibody responses comparable to 10 μg of the F1-V subunit vaccine with alhydrogel (Do et al. 2008). Although IgG1 and IgG2b isotypes were predominant in both the DEC-targeted and the F1-V subunit vaccine-treated group as described, the Th1-dependent IgG2a and IgG2c (C57BL/6) isotypes were observed only after the DEC-targeted LcrV immunization. This reflects the influence of Th1 cellular immunity induced by DC-targeting strategy.

In the beginning, two adjuvants were used in the DEC-targeting, combining both poly IC (50 μg) and αCD40 (25 μg) in a single dose of protein vaccine administration. Later, poly IC (50 μg) alone as an adjuvant with a prime and boost strategy was explored since this is a clinically more feasible approach (see Sect. 17.4.2 for more information). As before, broad and efficient LcrV-specific Th1 $CD4^+$ T cell responses were observed when LcrV protein was targeted to DEC-205^+ DCs in the presence of poly IC alone in a prime and boost strategy (Do et al. 2010). However, under these conditions, the antibody titers were less in comparison to standard F1-V subunit vaccine administration. For example, priming and boosting a mouse with 10 μg of αDEC:LcrV plus poly IC with 4–6 week separation generated tenfold lower titer when compared to those from priming and boosting a mouse with 10 μg of the F1-V subunit vaccine on alhydrogel. Nevertheless, Th1-dependent isotypes were still observed only in the DEC-targeted group as shown previously (Do et al. 2010).

In order to extend the DC-targeting strategy in the development of an improved pneumonic plague vaccine, LcrV protein was targeted to a new DC subset other than DEC-205^+ DCs, and then cellular and humoral immune responses compared. Distinct DC subsets have been characterized and, in the case of mice, there are two major myeloid DC subsets in spleen, based on the expression of CD8α and anatomic location (Shortman and Liu 2002). The DEC-205^+ DC subset expresses CD8α and the endocytic receptor, DEC-205, and is located in T cell zone within lymphoid organs. The $DCIR2^+$ DC subset does not express CD8α, but expresses another distinct uptake receptor, DCIR2, which can be recognized by anti-DCIR2 mAb. This subset is enriched in the bridging regions of the marginal zone (Dudziak et al. 2007). Previous studies showed that DEC-205^+ DCs are involved in three main activities: (1) uptake of dying cells (Iyoda et al. 2002); (2) resistance against certain viral infection (Allan et al. 2003); as well as, (3) cross-presentation (den Haan et al. 2000). For DEC-205^+ DCs, non-replicating antigens can be presented by MHC I, leading to CTL development, while $DCIR2^+$ DCs more rapidly form peptide MHC II complexes (Dudziak et al. 2007). Differences were observed between targeting of these two DC subtypes.

When LcrV was targeted to $DCIR2^+$ DCs via conjugated antibody in the presence of poly IC alone as an adjuvant, lower frequency of IFN-γ secreting T cells, but higher frequency of IL-4, IL-10, IL-13, or IL-5 secreting $CD4^+$ T cells were observed when compared to the DEC-205^+ DC targeted group. Moreover, about tenfold higher LcrV-specific antibody titers were observed in $DCIR2^+$ DC targeted group as compared to those induced by DEC-205^+ DC targeted group (Do et al. 2010). Thus, overall titers generated via prime-and-boosted with αDCIR2:LcrV

plus poly IC adjuvant were comparable to those generated by standard prime-and-boost with the F1-V subunit vaccine on alhydrogel. Immunoglobulin isotypes IgG1 and IgG2b predominated, and again Th1-depdent IgG2a isotypes were highly evident in each of the DEC- and DCIR2-targeted groups, while detection was difficult in the F1-V subunit vaccinated group. Although a previous report showed distinct pathways of T cell differentiation by distinct DC subsets under in vitro condition (Maldonado-Lopez et al. 1999), our study was the first to demonstrate differences in differentiation of $CD4^+$ T cell subsets in vivo leading to different antibody responses by distinct DC subsets. The functional aspects of the increased antibody titers induced by $DCIR2^+$ DC targeted LcrV proteins are further described in the Sect. 17.3.5.

17.3.4 Mucosal Immunity

The value of applying the DEC-targeting strategy as a novel mucosal vaccine against pneumonic plague has been tested. In contrast to pneumonias caused by other Gram-negative bacteria, such as *Klebsiella*, *Y. pestis* pathogenesis shows two distinct stages marked by a 48 h delay in recruitment of inflammatory cells to the lungs and appearance of proinflammatory cytokines and chemokines, indicating evasion, and/or suppression of host innate immune responses (Lathem et al. 2005; Bubeck et al. 2007). An adequately designed pneumonic plague vaccine should therefore prevent this delayed proinflammatory response so as to aid bacterial clearance before systemic dissemination. Various approaches have been examined to enhance mucosal immunity, including different immunization routes and adjuvants. For example, heterologous prime/boost strategies and intratracheal/intranasal administration of vaccines each enhanced mucosal immunity as compared to standard routes of administration (Eyles et al. 1998, 2000; Baca-Estrada et al. 2000; Reed and Martinez 2006). Cholera toxin B subunit, proteosome-based adjuvant (Protollin^{TM}), and the Toll-like receptor five agonist flagellin have all been tested in mucosal co-delivery with various F1/V subunit vaccines (Eyles et al. 1998; Honko et al. 2006; Jones et al. 2006). Antigens co-encapsulated in microspheres or formulated in liposomes induced superior humoral immunity at mucosal surfaces compared to free antigen delivery. In these cases, survival correlated with increased antibody titers in serum and lung washes (Eyles et al. 1998, 2000; Jones et al. 2006). Furthermore, intratracheal loading of anti-F1 or anti-V antibodies was sufficient to protect mice against aerosolized *Y. pestis* GB strain challenge (Hill et al. 2006), underscoring the correlation between high-antibody level and protection against *Y. pestis*. Clinical studies, however, are needed to show the induction of antigen-specific humoral and cellular immunity at mucosal surfaces with assay of correlates for protective efficacy.

Based on background, the efficacy of the DEC-targeting in the induction of LcrV-specific $CD4^+$ T cells at mucosal surfaces was first compared depending on administration routes. A single s.c. administration of the DEC-conjugated LcrV

with combined poly IC and αCD40 adjuvants induced LcrV-specific IFN-γ and IL-2 secreting $CD4^+$ T cells at mucosal surfaces, and the frequency of such T cells was increased with an i.n. route of administration (Do et al. 2012). When a prime and a boost strategy in the presence of only poly IC as adjuvant was applied to an intranasal route, again a strong and broad $CD4^+$ T cell response was induced. Three different mouse MHC haplotypes were tested [H-2^b(C57BL/6), H-2^d(BALB/c), H-2^k (C3H/HeJ)], primed and boosted in with αDEC:LcrV or isotype control in the presence of poly IC and lung samples evaluated for antigen-specific IFN-γ production by ICS. This prime-boost study identified two (C57BL/6 and C3H/HeJ) or three (BALB/c) T cell mimetopes that were broad as much as the previously described single dose of αDEC:LcrV + poly IC + αCD40 (Do et al. 2008). These data suggest that antigen processing by lung-associated DCs is similar to that of spleen DCs. Since DEC-205 KO (knock-out) mice produced no detectable antigen-specific $CD4^+$ T cell responses either by parenteral or intranasal routes, it is clear this effect was dependent on the expression of DEC-205 (Do et al. 2012). The mechanism of how DEC-targeting initiates immune responses at mucosal surfaces will be further investigated to better establish the rationale of future mucosal vaccine development. Although previous studies showed that most activated T cells migrated into non lymphoid tissues such as lung, liver, and kidney (Harris et al. 2002; Lefrancois and Puddington 2006), we show here that mucosal priming can induce local cell-mediated immunity. While the effector mechanisms of such protection are undefined, it is evident that enhanced antigen presentation by pulmonary DCs to resident and/or recruited memory—and naive T cells can be beneficial in vaccine design (Harris et al. 2002). Moreover, expression of inflammatory chemokines from effector T cells is critical for the early recruitment of supportive immune cells including immature DCs and natural killer cells (Lefrancois and Puddington 2006).

Different combinations of route in the prime/boost strategy were tested to compare the efficiency of mucosal immunity and found that interestingly priming s.c. and boosting i.n. induced higher frequency of IFN-γ secreting mucosal T cells when compared to s.c. priming and boosting (Do et al. 2012). It might be that an intranasal boost mimics a natural aerosol exposure to *Yersinia* and, hence, a mucosal boost would be preferable. Priming and boosting intranasally might be the optimal strategy for a vaccine as it induced the strongest antigen-specific cellular immunity at mucosal surfaces. It is advantageous that even after a single parenteral administration, the DEC-targeted protein could prime the immune system sufficiently enough to efficiently recall and activate antigen-specific T cells at mucosal surfaces (Do et al. 2012). Thus, it may be beneficial to develop the DEC-targeted protein vaccine with a mucosal delivery route to enhance T cell immunity at mucosal surfaces.

Regarding humoral immunity at mucosal surfaces, BAL (bronchoalveolar lavage) fluid was collected for comparison of immunoglobulin content (Do et al. 2012). The BAL fluid contained LcrV-specific antibodies from a single s.c. immunization with αDEC:LcrV + poly IC, but prime and boost immunization further enhanced the mucosal antibody responses tenfold. A broad spectrum of IgG

isotypes (IgG1, IgG2a, IgG2b) were detected following immunization with αDEC:LcrV, but IgG2a was not found in the BAL fluid from mice immunized with the F1-V subunit vaccine. Intranasal administration of the DEC-targeted LcrV protein increased the BAL antibody titers about 10 to 30-fold relative to the s.c. route. The isotypes of antibody were examined and IgG1 and 2b were consistently predominant while IgG2a isotype was the strongest in the DEC-targeted group. This concurs with other findings that protection against bacterial pneumonias is polarized for the IgG2a isotype (Corbeil et al. 1997; Mills et al. 1998). Interestingly, IgA responses were detected in BAL fluid following i.n. immunization but not with s.c. immunization. This suggests that local immunization is required to promote mucosal-specific antibodies such as IgA. Anti-LcrV IgG antibodies were found in the upper respiratory tract as well as in nasal washes both in the DEC-targeted and the F1-V subunit vaccine-treated group, but IgA responses in nasal washes were detectable only in F1-V plus alhydrogel immunized mice. In addition, after intranasal administration, serum anti-LcrV antibody titers were the highest in the F1-V plus alhydrogel immunized group, demonstrating again the superior efficacy of the F1-V subunit vaccine in the induction of humoral immunity.

17.3.5 Vaccine Efficacy: Challenge with Human Pathogen

For a challenge study using a virulent *Y. pestis* human pathogen, CO92 strain with intranasal inoculation of up to 25 μl 10^5 cfu (colony forming units) or ~100 LD_{50} was used to model the pneumonic form of plague. This is a high dose and a challenging method since *Y. pestis* CO92 requires special procedures as a CDC Category a agent. In case of the DEC-targeting strategy project, challenge experiments were performed at Public Health Research Institute (PHRI, Newark, NJ), a state of the art, licensed biological containment facility with approved security and safety procedures. All reagents for the vaccination were sent to the PHRI and thus, wherein the entire process from vaccination to challenge and related procedures were performed. In order to check proper priming before the challenge, random blood and spleen samples were submitted to the laboratory where LcrV-specific antibody titers and LcrV-specific $CD4^+$ T cell responses were confirmed by ELISA and ICS, respectively. After confirmation of the priming, intranasal challenge was performed and mice were observed twice a day for 14 days and the survival rate was reported.

The standard F1-V subunit vaccine with alhydrogel treatment was included as a positive control and a PBS treated group was included as a negative control. Various approaches were designed to test individual factors in conferring protection: (1) dosage, (2) targeting versus non-targeting, (3) administration routes, (4) adjuvants (poly IC vs. Ampligen, see more information in Sect. 17.4.2), as well as (5) heterologous priming and boosting strategy. Through all these efforts, the goal was to optimize the targeting strategy for protective efficacy for future clinical trials. The study was designed to answer the critical questions whether to induce combined

cellular and humoral immunity, to select a clinically acceptable adjuvant, and to demonstrate protective efficacy against pneumonic plague with a real human pathogen. Relevant findings were reported in the following two published papers.

In one study, the relative protection efficacy between two distinct DC subsets targeting was tested (Do et al. 2010). Groups of mice were primed and boosted intraperitoneally (i.p.) either with DEC- or DCIR2-targeted LcrV in the presence of poly IC at 4–6 weeks interval, then mice were challenged with 100 LD_{50} *Y. pestis* CO92 intranasally 6 weeks after the boost. F1-V + alhydrogel protected 100 % and αDCIR2:LcrV + poly IC protected 90 % of the mice, while αDEC:LcrV alone protected only 50 %. Interestingly, 50 % of mice survived after immunization with a single dose of αDCIR2:LcrV plus poly IC, as opposed to 0 % after a single dose of αDEC:LcrV. Similar findings were observed in a duplicate experiment and there was a clear correlation between protection and serum antibody levels.

In another study, protection efficacy of a mucosal DEC-targeted protein was tested as compared to a subcutaneous route of administration (Do et al. 2012). Mice were primed and boosted with αDEC:LcrV plus poly IC, either via an intranasal route or a subcutaneous route. Subcutaneous immunization with the F1-V subunit vaccine plus alhydrogel was included as a positive control. Six weeks after the boost, mice were inoculated intranasally with 100 LD_{50} of virulent *Y. pestis* CO92 strain. As expected, immunization with the F1-V conferred 100 % protection while all mice immunized with PBS did not survive the challenge. Interestingly, an intranasal route of αDEC:LcrV with poly IC protected 56 % of the mice while a subcutaneous route only protected 33 % of the mice. Moreover, a single intranasal priming with αDEC:LcrV with adjuvant protected 33 % while a single subcutaneous showed no impact on survival rate.

From these two studies, we have learned a few lessons in the development of plague vaccine. First, mice might not be the optimal animal model for testing the value of cellular immunity against pneumonic plague since we still find a strong correlation between survival rate and serum antibody titers. In particular, a high challenge dose such as 100 LD_{50} *Y. pestis* CO92 strain disguises the benefit of cellular immunity since mice cannot survive without antibodies regardless of cellular immunity induction. Further research using nonhuman primates may help discriminate the role for CMI given the fact that the F1-V subunit vaccine, which induces strong humoral immunity but poor cellular immunity, protects mice and cynomolgus macaques, but so far has failed to adequately protect African green monkeys, regardless of Ab titers measured by ELISA (Overheim et al. 2012a, b; Pitt 2004). Second, there appears to be a benefit by using the mucosal delivery of the DEC-targeted protein vaccine. Our findings show potential roles of enhanced mucosal T and B cell immunity induced by the mucosal delivery of the DEC-targeted protein vaccine when compared to a subcutaneous delivery group since serum antibody titers following vaccination were similar between these two groups (Do et al. 2012) (and unpublished data). A subsequent study will focus on demonstrating a more direct involvement of mucosal T and B cell immunity against pneumonic plague. In addition, further studies to investigate whether a mucosal delivery of $DCIR2^+$ DCs with LcrV protein or a combination therapy of distinct DC

subsets targeting (DEC-targeting + DCIR2-targeting) might improve the protection efficacy will provide informative knowledge of the DC-targeting strategy in plague vaccine development.

17.4 Lessons from DC-Targeting Strategy

As described above, the DC-targeting strategy has been under intensive investigation as an improved plague vaccine. Beyond this, the DC-targeting platform has also been applied to various other disease models, including infectious viral disease and cancer, to further explore unique features and efficacy. The potential of DC-targeting strategy as a novel prophylactic approach is described in Sect. 17.4.1. Although these preclinical data obtained from animal models provide background and rationale for future clinical applications, we understand there remains a large gap between experimental conditions and actual human disease. Therefore, it is necessary to make efforts to extend these findings to real human pathogenic situations, as described further in Sect. 17.4.3. In our estimation, such translational science begins with the selection of an adjuvant for the DC-targeting platform that is acceptable and well suited for human use (see Sect. 17.4.2 for more information).

17.4.1 Viral/Cancer Model

HIV/AIDS is a major global infectious disease that could benefit from the enhanced T cell immunity induced by the DC-targeting, or more specifically by the DEC-targeting strategy. Existing HIV/AIDS vaccines include prime and boost strategies with naked plasmid DNA and recombinant Vaccinia-Ankara and adenoviruses (Emini and Koff 2004). Since HIV-specific $CD4^+$ T cells help to produce functional HIV-specific $CD8^+$ T cells (Lichterfeld et al. 2004), and HIV patients with stronger $CD4^+$ T cell immunity have shown better clinical outcomes (Rosenberg et al. 1997), the DEC-targeting strategy has been adapted to explore an improved HIV/AIDS vaccine.

The Gag protein was selected as target for designing the anti-DEC-205 mAb due to previously known protective capacity in rhesus monkey studies as well as in clinical cases (Novitsky et al. 2003; Zuniga et al. 2006; Liu et al. 2009). Thus, gag p24 or gag p41 was conjugated to the DEC-targeting antibody. Initial tests used graded doses of αDEC:p24 or αDEC:p41, immunized in the presence of poly IC and αCD40 as adjuvants, and gag-specific T cell responses were measured by ICS following 6 h re-stimulation with peptide pools. This DEC-targeting method was 100 times more efficient for induction of gag-specific IFN-γ^+CD4^+ T cell responses as compared to a non-targeted soluble gag protein immunization (Trumpfheller et al. 2006). The DEC-targeting strategy was also superior to induce gag-specific IFN-γ^+CD4^+ T cell responses in comparison to other immunization strategies such

as a recombinant adenovirus-gag vaccine or prime-boost administration with a plasmid DNA vaccine. Similar to the plague vaccine studies (see above Sect. 17.3), the DEC-targeting strategy enhanced the breadth of gag-specific $CD4^+$ T cell responses such that two or three mimetopes were identified in different MHC haplotypes as well. Also verified in HIV model were the induction of long-term memory responses and mucosal resistance through a single s.c. immunization as described previously in OVA model. Briefly, even after 19–30 weeks (4 −7.5 months) after a single s.c. immunization of the DEC-targeted gag protein, enhanced effector memory $CD4^+$ T cells were induced which could proliferate and secrete IFN-γ upon re-challenge with reactive peptides. Importantly, neither control isotype nor the plasmid DNA vaccine achieved this response. In addition, mice vaccinated with the DEC-targeting gag protein plus adjuvants showed mucosal resistance against a challenge with recombinant vaccinia-gag, and such protection was ablated in IFN-γ receptor knockout mice or by depleting $CD4^+$ T cells (Trumpfheller et al. 2006).

This DEC-targeting strategy was further examined to discern the potential of using only poly IC as an adjuvant (Trumpfheller et al. 2008). Finding an optimal adjuvant with features, such as efficient immune stimulation while also being suitable for human use, is important in developing an improved vaccine (see Sect. 17.4.2 for more information). Thus, poly IC without αCD40 was tested independently since αCD40 is not clinically feasible. As a single s.c. or i.p. immunization, the DEC-targeting antibody with poly IC alone was found not to induce detectable antigen-specific IFN-γ secreting T cell responses. However, when applied in a 4 week prime-and-boost strategy, poly IC alone (50 μg/mouse/injection) induced ∼0.5–6 % of gag-specific IFN-γ^+CD4^+ T cell responses (Trumpfheller et al. 2008). Furthermore, the higher frequency of three cytokine (i.e., IFN-γ, IL-2, and TNF-α) producing gag-specific $CD4^+$ T cells was observed with this strategy as opposed to one or two cytokine producing $CD4^+$ T cells, and these T cells remained stable for 2–7 weeks after the boost. When vaccinia-gag was challenged via airway, the prime-and-boost DEC-targeting + poly IC again provided reasonable protection (Trumpfheller et al. 2008). Taken together, this study demonstrated the efficacy of the DEC-targeting strategy for the development of a novel therapeutic approach in HIV/AIDS vaccine through enhanced $CD4^+$ T cell immunity. In addition, these findings clearly showed the potential of poly IC as sole adjuvant in the DEC-targeting for clinically feasible application, specifically in a prime-boost regimen. This work therefore sets the stage for future development of various DEC-targeting strategies. Detailed data on the DEC-targeted gag protein in nonhuman primates and preclinical studies will be given in Sects. 17. 4.2 and 17.4.3.

Cancer is a complicated and challenging disease for developing a vaccine. Various factors must be considered together in order to achieve desired clinical outcomes, such as breaking the “self” tolerance of tumor antigens, overcoming the barriers of immune checkpoints, facilitating the accessibility of immune cells to the tumor beds, while at the same time homing treatment to the lymphoid tissues, and blocking tumor evasion mechanisms. Since successful cancer vaccination must satisfy these many constraints, it is reasonable strategy to combine more than one

approach. The DEC-targeting strategy has been examined to in the aim of solving any of these questions. Given the fact that there are fewer cancer phase trials applying T cell immunity compared to antibody-based immunity (i.e., Herceptin™ and Rituxan™), the demonstrated ability of the DEC-targeting to induce efficient T cell immunity makes it ready for application in the clinic. Ongoing efforts are described in below Sect. 17.4.3.

To enhance immunogenicity against tumor antigens, specific antigens are directly delivered to DCs in vivo via a DEC-targeting antibody. Survivin, mesothelin, or human epidermal growth factor receptor (HER2), were examined in three separate studies as tumor antigens targeted to the DCs. Survivin is non-mutated self-antigen which is expresses in thymus and regulates early thymocyte development (Okada et al. 2004). It shows anti-apoptotic activity, and overexpression of survivin is usually found in fast-growing transformed cell lines as well as in human cancer cells (Tamm et al. 1998; Islam et al. 2000; Grossman et al. 2001). Survivin enforces that the cell cycle to pass G2/M phase checkpoint, leading to oncogenesis (Li et al. 1998). There are correlations between survivin overexpression and unfavorable clinic outcomes (Islam et al. 2000; Altieri 2003; Blanc-Brude et al. 2003). Enhanced survivin-specific $CD4^+$ T cell immunity was reported in the DEC-targeting, especially when human survivin was conjugated to the mouse DEC-targeting antibody (Charalambous et al. 2006). Such a xenogeneic form of tumor antigen could enhance $CD4^+$ T cell immunity and, moreover, depletion of regulatory T cells (T_{reg}), further enhanced anti-tumor immunity (Charalambous et al. 2006). The significance of this study is the demonstration of strong $CD4^+$ T cell immunity to non-mutated self-antigen as induced by the DEC-targeting, which is otherwise rarely observed in other approaches.

Mesothelin is also found to be over-expressed in various human cancers, specifically, lung and pancreatic adenocarcinomas and ovarian cancers (Chang and Pastan 1996; Argani et al. 2001; Ho et al. 2007). Although biological functions of mesothelin need further study, it is known as a tumor differentiation antigen. Interestingly, it shows limited expression in normal tissues while being over-expressed in cancers as a non-mutated protein. For this reason, it has been useful therapeutic target in cancer immunotherapy (Bera and Pastan 2000; Hassan and Ho 2008). When pancreatic cancer patients were vaccinated with GM-CSF transduced allogenic pancreatic tumor cell lines, mesothelin was cross-presented, leading to the induction of cytotoxic T cell immunity (Thomas et al. 2004). This finding showed that our immune system could break the tolerance of "self" tumor antigens and induce anti-tumor immunity. In order to enhance such anti-tumor immunity, human mesothelin was conjugated to the mouse DEC-targeting antibody, and improved mesothelin-specific $CD4^+$ T cell immunity was observed (Wang et al. 2009). Moreover, antigen-specific IFN-γ^+CD8^+ T cell immune responses were observed by utilizing an ex vivo proliferating assay instead of the conventional (ICS) after 6 h re-stimulation with reactive peptides (Wang et al. 2009). The cross-presentation of proteins targeted via anti-DEC-205 fusion antibody is further described in Sect. 17.4.3.

HER2 is a tyrosine kinase growth factor receptor and its overexpression, which is closely associated with unfavorable clinical consequences, is found in 20–40 % of invasive breast carcinoma and about 70 % in ductal carcinoma patients (Slamon et al. 1987; Hynes and Stern 1994). Although improved diagnostic and therapeutic agents are utilized currently (i.e., Herceptin^{TM}), breast cancer still remains a high-mortality cancer in many countries. HER2 is a non-mutated self antigen and anti-HER2 T and B cell immunity has been reported in cancer patients (Disis et al. 1994, 2000). However, the soluble form of HER2 protein used as a vaccine is known to be inefficient in conferring protective immunity (Taylor et al. 1996; Dela Cruz et al. 2003). Various methods targeting HER2 to antigen presenting cells, including DCs, have also been tested. By utilizing B7-1/2 (Sloots et al. 2008), CD11c (Wei et al. 2009), CD40 (Kim et al. 2010), mannose (Thomann et al. 2011), or Fcγ receptor (Zizzari et al. 2011), the targeted HER2 could induce enhanced immunity when compared to non-targeted soluble protein treatments in mouse model. Development of breast cancer vaccine by utilizing the DEC-targeted HER2 protein represents one of these efforts, and its unique features (i.e., breadth, quality, and quantity of antigen-specific T cell immunity) described so far should be emphasized. When HER2 was targeted to the DEC-targeting antibody, plus poly IC as adjuvant, high quality, and the quantity of antigen-specific $CD4^+$ and $CD8^+$ T cells were induced (Wang et al. 2012). In particular, the HER2 specific $CD4^+$ T cells induced by the DEC-targeting could cross-react to Neu, a homologous rat protein. This implied "epitope spreading", which could be further exploited in protecting from other HER2 negative variants. Moreover, this cross-reaction allowed xeno-primed T cells (i.e., T cells induced by human HER2 targeted delivery) to be tested in trans-plantable neu-expressing tumor challenge model, where HER2-specific $CD8^+$ T cells played more important role in delaying tumor growth as well as lengthening the survivial rate (Wang et al. 2012). Taken together, this novel approach is expected to be further tested in cancer patients.

17.4.2 Adjuvants

The combined poly IC (polyinosinic:polycytidylic) and αCD40 was used as a combined adjuvant in initial trials of the DEC-targeting strategy (Bonifaz et al. 2004; Charalambous et al. 2006; Trumpfheller et al. 2006; Do et al. 2008). Given that αCD40 cannot be used in clinical setting, due to the concordant induction of massive apoptotic death of immune cells, the efforts to utilize poly IC alone in a prime and boost strategy were previously explored. Poly IC is a synthetic double strand RNA that mimics viral infection and delivers signal through Toll-like receptor (TLR) 3 (Alexopoulou et al. 2001; Kawai and Akira 2007). A main reason to choose poly IC as an adjuvant in the DEC-targeting strategy is that it is clinically acceptable adjuvant, and it has been used in cancer and HIV patients with efficacy by secreting large amount of interferon mimicking viral infection (Krown et al. 1985; Lampkin et al. 1985; Stevenson et al. 1985; Salazar et al. 1996). Another important feature is that

poly IC effectively induces cellular immunity. In the mouse model, various TLR ligands such as MALP-2 (for TLR2/6), Pam3Cys (for TLR1/2), LPS (for TLR4), R-848 (for TLR7/8), CpG (for TLR9), or poly IC (for TLR3) were tested as adjuvant in the DEC-targeting antibody immunization (Longhi et al. 2009). The data showed that poly IC was the superior adjuvant in the induction of antigen-specific $CD4^+$ T cell responses. Section 17.5.2 contains more information on the proposed mechanism. The efficacy of poly IC for inducing T cell immunity was further confirmed in non-human primate studies (Stahl-Hennig et al. 2009). Unlike mice, poly IC is not a strong inducer of type I IFN-α in primates and it might be due to its fast degradation by serum nucleases which are known to be more abundant in primates than in rodents (Nordlund et al. 1970). Thus, naked poly IC was examined alongside poly ICLC (poly IC with poly-L-lysine and carboxymethylcellulose), which is 5–10 times more resistant to RNAase hydrolysis in primate serum and it showed a large amount of type I IFN induction (Levy et al. 1976; Stephen et al. 1977). Briefly, rhesus macaques were immunized s.c. either with KLH or a low dose (10 μg/animal) of poor immunogenic human papillomavirus (HPV)16 capsomers in the presence of either poly IC, poly ICLC, or CpG-C as adjuvant. Although both poly IC and poly ICLC could induce antigen-specific T cell immunity, the highest proliferative responses were observed with 2 mg/animal of poly ICLC treatment (Stahl-Hennig et al. 2009). Moreover, similar dose of CpG-C, a control adjuvant, in similar aqueous solution (not in water-in-oil emulsion) as poly ICLC did not induce HPV16-specific T cell immunity while together they did induce antibody responses (Stahl-Hennig et al. 2009). This data demonstrates that poly IC (or poly ICLC) can be used in primates as an effective adjuvant, particularly for inducing antigen-specific cellular immunity, which might benefit an improved vaccine. In contrast, two well-known approved adjuvants in humans, alum and monophosphoryl lipid A (MPLA), are known to be limited in their capacity to induce cellular immunity.

Recently, interesting results were obtained in healthy volunteers using a subcutaneous route of poly ICLC, contrary to not the intramuscular (i.m.) route as previously employed (Caskey et al. 2011). First, a single s.c. dose of 1.6 mg poly ICLC was well tolerated in phase I studies. Secondly, transcription profiling, using RNAs from whole blood samples obtained from the volunteers before and after the poly ICLC vaccination, showed the up-regulation of genes involved in many different innate immunity pathways, such as IFN-regulated genes, inflammasome-associated genes, NF-κB, DC maturation and antigen-presentation (Caskey et al. 2011). Interestingly, such profiling was very similar to and overlapped with that of attenuated yellow fever viral vaccine YF17D (Caskey et al. 2011), a successful vaccine proven in humans and showing the efficacy of poly IC as a true viral mimic adjuvant.

Poly $IC_{12}U$ (Ampligen^{TM}) is another modified form of poly IC which shows instability in serum, resulting in low toxicity in humans, and thus, has been considered a suitable adjuvant for human vaccines (Thompson et al. 1996; Ichinohe et al. 2007). As mentioned in the nonhuman primate study, poly $IC_{12}U$ also induced antigen-specific T cell immunity, but it required higher dose (6 mg/animal) to show similar effect as poly ICLC (2 mg/animal) (Stahl-Hennig et al. 2009). However, when it was used as an adjuvant in the DEC-targeting strategy in the development

of plague vaccine (see Sect. 17.3 for more information), we observed neither adjuvant activity nor protective efficacy (Do unpublished data).

Poly IC was also tested in a mucosal delivery of the DEC-targeting strategy in the development of plague vaccine (see Sect. 17.3.4 for more information) (Do et al. 2012). Although poly IC had been utilized as a mucosal adjuvant before, most of studies showed an adjuvant effect by poly IC in inducing antibody responses in non-targeted protein vaccine or inactivated viral vaccine (Ichinohe et al. 2005; Partidos et al. 2005). However, in the DEC-targeting strategy, poly IC particularly demonstrated the induction of enhanced cellular and humoral immunity at mucosal surfaces (Do et al. 2012). Moreover, when other adjuvants such as LPS (lipopolysaccharide) from *Escherichia coli* 055:B5 were used as a mucosal delivery of the DEC-targeting, it showed tenfold weaker activity in inducing Th1 immunity as compared to poly IC. Another frequently used adjuvant, the TLR5 agonist flagellin, did not induce pulmonary cellular immunity, either with the DEC-targeted vaccine or F1-V subunit vaccine treatments (Do et al. 2012) (data not shown).

Beyond dsRNA, TLR4 agonists have also been recently tested as adjuvants in the DEC-targeting strategy (Pantel et al. 2012). LPS is a well-known TLR4 agonist and induces DC maturation as well as strong humoral immune responses (Steinhagen et al. 2011). To reduce toxicity while maintaining immunogenicity of the original LPS, chemically modified versions were developed for use as vaccine adjuvants. MPLA, a low-toxicity derivative of lipid A from *Salmonella minnesota* R595 lipopolysaccharide, is one derivative that has gained popular use for this purpose (Mata-Haro et al. 2007). MPLA in alum is currently being used as adjuvant in hepatitis B and human papilloma vaccine (Dubensky and Reed 2010). Glucopyranosyl lipid A (GLA) is a new synthetic TLR4 agonist and already demonstrated to be safe during phase I trial with Fluzone influenza vaccine (Coler et al. 2010). A stable oil-in-water emulsion called GLA-SE has shown enhanced antibody responses as well as Th1-type cellular responses in mouse and cynomolgus monkey models (Baldwin et al. 2009; Coler et al. 2010). Interestingly, when GLA-SE was included in the DEC-targeting strategy, it induced DC maturation in vivo as early as 4 h after immunization, demonstrating its utility as a novel adjuvant in the induction of antigen-specific antibody responses and Th1-type cellular immunity (Pantel et al. 2012). Altogether, these findings establish that clinically feasible TLR3 and TLR4 agonists can be incorporated into the DEC-targeting strategy for inducing enhanced antigen-specific humoral and cellular immunity. Continuous efforts on exploring improved adjuvants optimal for the DEC-targeting strategy should be encouraged.

17.4.3 Pre-clinical Study

Various efforts have been applied to extend the DEC-targeting strategy into clinical testing. Some of the previous studies utilized human instead of mouse antigen in the DEC-targeting strategy, and such targeted xenogenic protein enhanced overall immunogenicity. Broad human antigen-specific $CD4^+$ T cell immunity on various

MHC haplotypes was observed with human antigen peptides pools as well (Charalambous et al. 2006; Wang et al. 2009, 2012).

Anti-human DEC-205 targeting antibody was also generated and tested for comparison to anti-mouse DEC-205 targeting antibody (Guo et al. 2000). In contrast to the mouse counterpart, human DEC-205 is a transmembrane protein with 10 C-type lectin domains having 77 % gene sequence homology (Jiang et al. 1995). Anti-human DEC-205 targeting antibody has been utilized in various settings to gauge its potential for clinical application. The DEC-targeting antibody was found to be superior for inducing antigen-specific IFN-γ secreting $CD8^+$ T cell responses when PBMCs (peripheral blood mononuclear cells) or PBMC-derived DCs and T cells from HIV-infected patients were targeted with HIV gag p24 protein through either anti-human DEC:p24 (αhDEC:p24), αhDC-SIGN:p24, αhMMR:p24, or isotype control antibodies (Bozzacco et al. 2007). In addition, broad $CD8^+$ T cell responses were observed with the DEC-targeting approach. Specifically, eight different gag peptides were identified from 11 patient samples after injection of αhDEC:p24 targeted protein (Bozzacco et al. 2007). These data suggest that through DEC-205 targeting, DCs can enhance cross-presentation of HIV gag p24 protein to broad gag-specific $CD8^+$ T cells leading to IFN-γ secretion. Considering naturally high polymorphic MHC products in human antigen presentation, these results underscore the efficiency and the potential of the DEC-targeting strategy as a novel protein-based vaccine in HIV/AIDS.

In further studies, two novel mouse systems were utilized in demonstrating human DEC-205 targeting strategy as an effective vaccine approach. First, human DEC-205 targeted antibody (MG38.2 Ab) (Guo et al. 2000) was conjugated with nuclear antigen one of Epstein-Bar-virus (EBV, EBNA1) and used as a vaccine against both EBV primary infection and EBV-associated malignancies (Gurer et al. 2008). Briefly, 2–5 days old NOD/LtSz-*scid IL2R*γ^{null} (NOG) mice were irradiated and injected with $CD34^+$ human hematopoietic stem cells obtained from human fetal liver. Successful reconstitution was confirmed in the mice after 10–12 weeks and the mice were primed and boosted with αhDEC:EBNA1 in the presence of poly IC as an adjuvant. Two months after the DEC-targeting, EBNA1-specific IFN-γ^+CD4^+ T cells and antibody responses were observed (Gurer et al. 2008). In another study, a transgenic mouse expressing human DEC-205 receptor on mouse $CD11c^{hi}$ DCs was generated and cellular and humoral immune responses induced by a newly synthesized anti-human DEC-205 targeting antibody (3G9 mAb) conjugated with HIV gag p24 protein were examined (Cheong et al. 2010). Again the DEC-targeting showed enhanced antigen-specific $CD4^+$ T cell responses when compared to non-targeted or DC-SIGN-targeted protein approach, as mentioned above (Bozzacco et al. 2007). In addition, the ability for cross-presentation of the DEC-targeting antibody was confirmed where HIV gag-specific $CD8^+$ T cells from both long-term nonprogressor patient and chronically HIV-infected patient proliferated and secreted significant amount of IFN-γ (Cheong et al. 2010). Thus, efficient cross-presentation was demonstrated in the DEC-targeting delivery of antigen.

This newly synthesized anti-human DEC-205 targeting antibody (3G9 mAb) was examined in nonhuman primates as well (Flynn et al. 2011). A heterologous

prime-boost strategy, known to increase immunity when compared to a homologous prime-boost regimen, was investigated via the DEC-targeting strategy. Rhesus macaques were primed s.c. three times either with 60 μg of the non-targeted HIV gag p24 protein or the DEC-targeted gag p24 in the presence of 1 mg of poly ICLC at 0, 8, and 27 weeks. All animals were s.c. boosted with New York vaccinia virus (NYVAC) containing HIV Gag/Pol/Nef (Gomez et al. 2007) at 31 weeks after the protein priming (i.e., at 58 weeks). Priming alone with NYVAC-HIV Gag/Pol/Nef as a control group induced low to undetectable gag-specific T cell responses but showed enhanced cellular immunity after boost. Either non-targeted or DEC-targeted HIV gag p24 protein could induce gag-specific $CD4^+$ and $CD8^+$ T cell responses as well as antibody responses (Flynn et al. 2011). However, two interesting and valuable results could be drawn from this study regarding the DEC-targeting strategy. In comparison to non-targeted immunization, the DEC-targeting approach was more effective in cross-presentation as well as in generating multi-functional $CD4^+$ and $CD8^+$ T cells. In particular, when isolated $CD8^+$ T cells from the animals were examined for proliferative capacity, for multi-cytokine secretion (i.e., IFN-γ, IL-2, and TNF-α), and for long-term memory responses, the DEC-targeting showed enhanced efficacy in all aspects in comparison to non-targeted protein vaccine (Flynn et al. 2011). Such multi-functional T cells correlated with better protection against *Mycobacterium tuberculosis*, *Leishmania major*, and vaccinia virus in mouse models (Trumpfheller et al. 2006; Darrah et al. 2007; Lindenstrom et al. 2009).

The findings from nonclinical testing including nonhuman primate studies have clearly demonstrated the efficacy of the DEC-targeting as a novel protein vaccine useful for future clinical application. To this objective, a phase I study to evaluate the safety and immunogenicity of the DEC-targeted HIV gag p24 protein with poly ICLC is on-going with 45 healthy volunteers aged 18–60 at The Rockefeller University Hospital (Unpublished data: Personal communication with and oral presentation by Dr. Trumpfheller C., at the 12th International Symposium on Dendritic Cells, October 7–11, 2012, Daegu, Korea). To date, antigen-specific cellular and humoral immune responses with low and transient reactogenicity were reported and additional valuable results are anticipated.

17.5 Summary: Future Direction for DC-Targeting Strategy

In this chapter, we have described the unique features, efficacy, and benefits of the DC-targeting vaccine strategy in various disease models and summarized data that demonstrate it to be an efficient and novel vaccine approach, ready for translation to clinical testing for treating human disease. Beyond the examples introduced in Sect. 17.4.3 as preclinical efforts, the following comments highlight important areas of focus for future investigation in further development of DC-targeting as a viable human vaccine platform.

17.5.1 Human DC Subsets and Targeting

Human DC subsets and mouse DC subsets are not identical (Palucka et al. 2010). This means that a better understanding of human DC subsets is required to extend the DC-targeting strategy into clinical application. Instead of addressing the diversity and biology of human DC subsets in detail, we instead focus here on DEC-205$^+$ DCs which are the human equivalent of mouse CD8α$^+$ DCs (Palucka et al. 2010; Villadangos and Shortman 2010). Previous studies suggested that CD141$^+$ human DCs (also known as BDCA3$^+$ DCs) might be the counterpart of mouse CD8α$^+$ DCs based on four similarities: (1) the expression of chemokine receptor XCR1 (Bachem et al. 2010); (2) the expression of transcription factors Batf3, and IRF-8, and a lack of IRF-4; (3) the expression of TLR3, but not TLR7 and the secretion of IL-12 upon activating through TLR3; and (4) the capacity of phagocytosis of dead cells and cross-presentation (Jongbloed et al. 2010; Poulin et al. 2010). However, differences still exist. For example, CD141$^+$ DCs do not express TLR9 although CD8α$^+$ DCs do, and different signaling is required for IL-12 production from CD141$^+$ DCs as compared to mouse CD8α$^+$ DCs (Jongbloed et al. 2010; Poulin et al. 2010). In addition, the ability of DC cross-presentation in mouse is known to be strictly limited to CD8α$^+$ DCs (Hildner et al. 2008; Lin et al. 2008), but human DC subsets employ more than just CD141$^+$ DCs for cross-presentation (Schnorrer et al. 2006; Segura et al. 2009). The hurdles of characterizing human equivalents of mouse DC subsets arise not only from species intrinsic discrepancy, i.e., anatomical differences and developmental process so that few markers are shared between human and mouse DCs, but also from a paucity of relevant human lymphoid DC supply, caused by ethical and logistical difficulties faced over the past decade.

Previously, human DC subsets principally in lymphoid organs were investigated (Granelli-Piperno et al. 2005). This aligned with our interest in using DEC-205$^+$ DCs for future DC-targeting strategy vaccines. In addition, these cells are found to be abundant in T-cell areas of human lymph nodes and they co-express CD11c as well as MHC class II, both critical for effective targeting. In the case of human spleen, another study showed that CD11c$^+$ DCs were located in T cell-, B cell-, and marginal zones, and most were found to be immature by lacking CD86 and CD83 receptors (McIlroy et al. 2001). When we used CD205 (DEC-205) to identify human spleen DCs, we found that most DEC-205$^+$ DCs are CD11c$^+$ and immature, consistent with the prior observation (Pack et al. 2008). Interestingly, in addition to known tropism for white pulp, these DEC-205$^+$ DCs were closely associated with cells displaying the mucosal address in cell adhesion molecule, MAdCAM. Moreover, human spleen DEC-205$^+$ DCs were found both in T cell- and non-T cell areas, unlike mouse splenic counterparts found only in T-cell areas. This unexpected finding warrants further investigation to define the natural localization of DEC-205$^+$ DCs. Such differences underlie the need to perform efficacy studies of DEC-targeting strategy using human DC subsets, and findings will guide the design of a fully human DC-targeting strategy. This information is critical since DCs exist in distinct functional states depending on activation/maturation signals derived from

microenvironments, such as infectious microbes, tissue-derived cytokines, and other immune cells, which controls the target, timing, and quality of adaptive immunity. Multiple parameters should be considered in designing human DC-targeting strategy, including the desired type of immune protection to be induced (cellular vs. humoral immunity), the tissue distribution and receptors of various DC subsets, and activation signals based on TLRs expressed for optimal choice of adjuvant.

17.5.2 Mechanism

While ample publications show the efficacy of the DEC-targeting strategy in various disease models, few reports address the basic mechanisms at work in DC-targeting. Two studies in particular are worth noting here as leads for further investigation to uncover downstream mechanisms underlying an improved DC-targeting strategy.

The first study investigated how poly IC works as an effective adjuvant for DEC-targeting. As shown in Sect. 17.4.2, poly IC was found to be a superior adjuvant for DEC-targeting in inducing Th1 type cellular immunity as compared to other known TLR ligands (Longhi et al. 2009). Poly IC was shown to be the most effective inducer of type I interferon (IFN) through mda5 (melanoma differentiation-associated protein 5) and TLR3. A blocking experiment showed that type I IFN was in fact essential for DC maturation and $CD4^+$ T cell development. Specifically, type I IFN acted in vivo directly on DCs, leading to up-regulation of DC co-stimulatory molecules, and DCs exposed to type I IFN became better inducers of mixed lymphocyte reactions. In addition, poly IC induced Th1 $CD4^+$ T cell responses primarily through type I IFN, but interestingly type II IFN (IFN-γ) or IL-12 were not necessary. Lastly, bone-marrow chimera experiments demonstrated that the main in vivo sources of type I IFN were nonhematopoietic radio-resistant cells as well as monocytes and DEC-205^+ DCs, and the adjuvant effect of poly IC was more prominent when systemic rather than local inflammatory responses were induced (Longhi et al. 2009).

The second study examined the influence of DC subsets on antigen processing. DCs express different antigen uptake receptors, and, to date, we utilized DEC-205 receptor for $CD8\alpha^+$ DC-targeting and DCIR2 receptor for $CD8\alpha^-$ DC-targeting. Regarding the OVA model antigen, a previous study demonstrated enhanced MHC class II presentation by targeting $DCIR2^+$ rather than DEC^+ DCs (Dudziak et al. 2007). This difference in antigen processing was shown to be intrinsic to the DC subsets, and not due to differences in receptors targeted by anti-DEC-205 or 33D1 (DCIR2) mAbs. The data showed that the enhanced antigen presentation on MHC class II by $DCIR2^+$ DCs was associated with increased expression of proteins involved in MHC class II processing, such as cathepsin (Dudziak et al. 2007). Moreover, as shown in Sect. 17.3.3, when real human pathogenic antigen (*Y. pestis* LcrV) was utilized in distinct DC subsets targeting, enhanced humoral immunity was observed with targeting $DCIR2^+$ DC (Do et al. 2010). An extensive follow-on

study is under way to reveal downstream mechanisms on which distinct DC subsets induce different type of adaptive immunity (Do, manuscript in preparation).

While these studies are a useful beginning, further investigation of various aspects, including at molecular levels, are required for improved DC-targeting strategy as a novel vaccine approach.

17.5.3 Construct Limitation and Antigen Selection

The DC-targeting strategy highlighted in this chapter uses a recombinant conjugated antibody construct. This genetically engineered antibody conjugate has distinct advantages as already described above. However, important aspects of this technology require case-by-case characterization to support clinical application. First and foremost is the necessity of identifying an effective target antigen. This is a challenge not only for the DC-targeting strategy field but also for general vaccine development, especially for vaccines of noninfectious diseases. Second is the actuality that recombinant construct will not always function as intended. Not every construct of the conjugated antibody will be expressed well and successfully induce immunity. Molecular properties such as size, conformation, solubility, and stability of the recombinant antigen-antibody are not yet well predicted by modeling, thus creating need for empirical testing to determine yield and efficacy of the recombinant conjugated antibody. To date, the 293 T cell transient transfection systems has been employed with good result for such engineering (see Sect. 17.6 for more information), but unknown biases may be revealed through exploration of other expression systems. Although these hurdles need to be addressed for improvement of the technology, we have established the great utility of recombinant antibody-antigen vehicle for the DC-targeting vaccine platform. Further applications, for example, combining with other drug delivery systems such as nanoparticles or developing novel technique for efficient screening system of newly synthesized antigen-antibody construct should be encouraged for refining the DC-targeting strategy.

17.6 Generalized Method

Following are general guidelines for creating a DC-targeting strategy to any antigen of interest. The reader is advised to inspect corresponding sections of this chapter and references therein for greater details at each stage of method engineering and testing.

17.6.1 Construct of Antigen-Conjugated DC-Targeting Antibody

The full length coding region of the antigen gene is amplified and then cloned into the COOH terminus of heavy chain of anti-DEC-205 mAb, anti-DCIR2 mAb, or an isotype control mAb. Each construct is transiently transfected into 293 T cells and the expressed conjugated antibody is purified by protein G affinity chromatography. The purified, recombinantly conjugated antibody is then characterized for structure and quality by SDS-PAGE and Western Blotting, and for binding affinity and specificity by FACS (fluorescence activated cell sorting) using Chinese hamster ovary (CHO) cells which have been stably transfected with respective receptors (i.e., DEC-205/DCIR2). It is important to insure that all reagents are free of endotoxin.

17.6.2 Immunization

Each conjugated antibody (5–10 μg/mouse or indicated dosage), with or without a stimulus for DC maturation, is administered subcutaneously (s.c.), intraperitoneally (i.p.), or intranasally (i.n.) depending on the study. DC stimulating reagents that we have found to be successful include anti-CD40, poly IC, or a combination of anti-CD40 and poly IC. In case of using poly IC alone as an adjuvant, a prime and boost strategy is necessary.

17.6.3 Verification of Antigen-Specific T Cell Induction

Antigen-specific T cell responses are analyzed by either ICS, ELISPOT (enzyme-linked immunosorbent spot assay), or by CFSE (Carboxyfluorescein succinimidyl ester)-proliferation assay. Briefly, bulk splenocytes are stimulated with reactive peptide pools (2 μg/ml or indicated concentration) or control medium in the presence of a co-stimulatory anti-CD28 mAb for 6 h. Brefeldin A is added for last 4–5 h to accumulate intracellular cytokines. After stimulation, cells are collected, washed, and stained with various surface antibodies (i.e., CD3/CD4/CD8). Following fixation and permeabilization, cells are incubated with various anti-cytokine antibodies (i.e., IFN-γ, IL-2, TNF-α). Binding specificities are collected by flow cytometer and analyzed (i.e., FlowJo software).

For ELISPOT assays, specialized ELISPOT plates are coated overnight with purified capturing various anti-cytokine antibodies (i.e., IFN-γ, IL-2, TNF-α). The plates are washed, blocked, and then incubated with sorted T cells ($CD4^+$ or $CD8^+$), purified spleen $CD11c^+$ DCs, and in the presence or absence of reactive peptides. After 48 h, the plates are washed and incubated with proper detecting anti-cytokine antibodies and then the spots are visualized and counted.

For CFSE-proliferation assay, bulk splenocytes are labeled with CFSE and the labeled cells are stimulated with or without reactive peptides for 4 days. This can be combined with ICS for the last 6 h of culture. Data are collected by flow cytometer and analyzed.

17.6.4 Verification of Antigen-Specific B Cell Induction

Antigen-specific antibody responses are measured by either ELISA or ELISPOT. Briefly, specialized ELISA plates are coated with respective antigens overnight. Plates are washed, blocked, and placed with serial diluted samples such as serum or bronchoalveolar lavage fluid. Various secondary detecting antibodies (i.e., IgG, IgA) are added and visualized by spectrophotometer and titers are calculated.

For antibody-secreting cell detection by ELISPOT, specialized ELISPOT plates are coated with antigens of interest overnight. The plates are washed, blocked, and then placed with serial diluted bulk splenocytes or bone-marrow cells. After 6 h, the plates are washed and incubated with respective secondary detecting antibodies (i.e., IgG, IgA) and the spots are visualized and counted.

17.6.5 Vaccine Efficacy Tests

The efficacy of conjugated Abs can be evaluated either as a therapeutic or a prophylactic vaccine. Protocol will be different depending on disease models. For example, as a therapeutic vaccine in cancer model, mice are first inoculated with cancers and then treated with various vaccine regimens. In contrast, as a prophylactic vaccine in the cancer model, mice are first injected with various vaccine regimens and then they are challenged with tumors. In either case, tumor volumes, and survival rates are measured and the efficacy is interpreted. The nature of pathogenesis and historical vaccination will determine the study methods to be used measuring effectiveness against a targeted infectious disease (for example, see Sect. 17.3 on vaccination against plague).

17.7 Considerations for Analytical Testing

Nonclinical analytical testing and clinical studies document that a biological drug product meets prescribed specifications for safety, purity and potency, as is required by 21 CFR parts 600.3(s), 601 and 312, and ICH Q5C. However, conventional DC-targeting vaccines are classified as medical device and have somewhat relaxed requirements. Table 17.1 lists analytical tests that have been used in demonstrating intended attributes of DC vaccines as compared to those used for conventional DC vaccines, which require more testing.

Table 17.1 Summary and comparison of analytical assays used in DC vaccines

Stage	Analytical tests	Purpose
Dendritic cell targeting strategy (*experimental mouse model*)		
DC targeting antibody production	Coomassie staining and western blotting	Antibody integrity check
	Biuret assay	Antibody concentration check
	Flow cytometry with CHO (Chinese Hamster Ovarian) transfectant cells expressing DEC-205 or DCIR2 receptor	Antibody receptor binding specificity & binding strength check
	Endotoxin test	Antibody purity check
Immune response read-out	T cell proliferation T cell cytokine production T cell functional assay	Antigen-specific T cell response check
	Antibody quantification Antibody neutralization	Antigen-specific B cell response check
	Various in vivo assays	Functional aspects of antigen-specific immune responses[e]
Conventional Dendritic cell-based vaccine (*experimental mouse model*)		
DC precursor generation	Flow cytometry	DC characterization
	Phagocytosis	Antigen uptake capacity test
	Microscopy observation	DC morphology check
Antigen pulsing	Antigen-specific T cell assay	Antigen presentation capacity test[a]
	Flow cytometry	Antigen presentation capacity test[b]
DC maturation	Flow cytometry	Matured DC characterization[c]
	DC cytokine production	Matured DC characterization
	Phagocytosis	Matured DC characterization[d]
	Migration assay	Matured DC characterization
	Mixed lymphocyte reaction	Matured DC characterization
Immune response read-out	T cell proliferation	Antigen-specific T cell response check
	T cell cytokine production	
	T cell functional assay	
	Antibody quantification	Antigen-specific B cell response check
	Antibody neutralization	
	Various in vivo assays	Functional aspects of antigen-specific immune responses[e]

[a] various DC:T cell ratio should be considered
[b] in case of anti-antigen-antibody is available
[c] some of surface markers are up-regulated or down-regulated when compared to immature form of DCs
[d] matured DCs down-regulated phagocytosis when compared to immature form of DCs
[e] for example, vaccine efficacy test

Table 17.2 List of all DC vaccine clinical trails

Identifier	Title	State number
NCT00001566	A pilot study of autologous T-cell transplantation with vaccine driven expansion of anti-tumor effectors after cytoreductive therapy in metastatic pediatric sarcomas	MD8
NCT00001827	p53 vaccine for ovarian cancer	MD9
NCT00003229	Vaccine therapy in treating patients with metastatic melanoma who are undergoing surgery for lymph node and tumor removal	NC26
NCT00003432	Immunotherapy in treating patients with metastatic breast cancer	NC23
NCT00003433	Immunotherapy in treating patients with resected liver metastases from colon cancer	NC24
NCT00003665	Vaccine therapy in treating patients with stage IV melanoma	PA8
NCT00003792	Vaccine therapy in treating patients with metastatic melanoma	TX17
NCT00003977	Vaccine therapy in treating patients with recurrent or persistent cervical cancer	MA20
NCT00004025	Vaccine therapy with or without interleukin-2 in treating patients with stage III or stage IV melanoma	MA13, TX8
NCT00004211	Vaccine therapy in treating patients with metastatic prostate cancer	NC4
NCT00004604	Biological therapy in treating patients with metastatic cancer	NC22
NCT00004880	Vaccine therapy in treating patients with advanced kidney cancer	CA4
NCT00005617	Vaccine therapy in treating patients with stage iv or relapsed malignant melanoma	CA29
NCT00005816	Vaccine therapy in treating patients with stage III or stage IV kidney cancer	NC16
NCT00005947	Vaccine therapy in treating patients with metastatic prostate cancer that has not responded to hormone therapy	FL11, MN4, NJ3, NY14, OH7, OR4, PA24, TX13, WA6
NCT00005992	Vaccine therapy in treating patients with metastatic prostate cancer that has not responded to hormone therapy	CA27, TX9
NCT00006113	Vaccine therapy followed by biological therapy in treating patients with Stage III or stage IV melanoma	CA14
NCT00006434	Tumor lysate pulsed-dendritic cell vaccines after high-dose chemotherapy for non-hodgkin's lymphoma	MI2
NCT00010127	Vaccine therapy in treating patients with metastatic prostate cancer	NC18

(continued)

Table 17.2 (continued)

Identifier	Title	State number
NCT00012064	Vaccine therapy in treating patients with stage IV or recurrent melanoma	CA17
NCT00013572	HIV candidate vaccine, ALVAC-HIV-1, administration in HIV-negative adults	MD10
NCT00014131	Vaccine therapy in treating patients with kidney cancer	CA20
NCT00017355	Vaccine therapy in treating patients with metastatic melanoma	TX10
NCT00019084	Vaccine therapy and biological therapy in treating patients with advanced cancer	MD6
NCT00019214	Vaccine therapy with or without interleukin-2 in treating patients with metastatic melanoma	MD17
NCT00019591	Vaccine therapy with or without interleukin-2 in treating patients with locally advanced or metastatic colorectal cancer	MD12, MD13, TN4
NCT00019929	Vaccine therapy in treating patients with stage III non-small cell lung cancer	MD5, TN1
NCT00022334	Vaccine therapy in treating patients with liver cancer	CA40
NCT00023985	Vaccine therapy in treating patients with non-small cell lung cancer	NY9
NCT00026624	Safety/immunogenicity of immunizations of ALVAC-DC-SC versus ALVAC-SC	MA14, NY12
NCT00027534	Vaccine therapy in treating patients with advanced or metastatic cancer	NC11
NCT00039325	Vaccine therapy in treating patients with stage IV or recurrent malignant melanoma	CA22
NCT00045968	Study of a Drug [DCVax®-L] to treat newly diagnosed GBM brain cancer	CO3, DC4, FL12, IL5, IN4, MA17, MI5, MN5, MO3, NJ4, NY15, OH5, SC4, TN3, TX15, WA7
NCT00049218	Chemotherapy followed by vaccine therapy in treating patients with extensive-stage small cell lung cancer	FL7
NCT00050323	Safety/efficacy of a vaccine prepared from dendritic cells combined with tumor cells to treat advanced kidney cancer	CA35, MA16, OH6
NCT00053391	Vaccine therapy in treating patients with stage III or stage IV melanoma	GERMANY4
NCT00056134	Vaccine therapy in treating patients with stage III or stage IV melanoma	GERMANY3
NCT00056758	HIV vaccine designed for HIV infected adults taking anti-HIV drugs	PA19

(continued)

Table 17.2 (continued)

Identifier	Title	State number
NCT00057915	Vaccine therapy in treating patients with refractory stage IV cancer	NC17
NCT00058734	Therapeutic vaccination followed by treatment interruption in HIV infected patients	MA18
NCT00065442	Provenge® (Sipuleucel-T) active cellular immunotherapy treatment of metastatic prostate cancer after failing hormone therapy	CO2, DC3, FL10, GA3, IL4, IN3, MD11, MA15, MN3, NJ2, NY13, NC19, OH4, OR3, PA23, SC3, TX12, WA4, WI2, CANADA2
NCT00068510	Vaccine therapy in treating patients with malignant glioma	CA31
NCT00074230	Vaccine therapy in treating patients with stage IV cutaneous melanoma	GERMANY2
NCT00082641	Vaccine therapy with either neoadjuvant or adjuvant chemotherapy and adjuvant radiation therapy in treating women with p53-overexpressing stage III breast cancer	NE2
NCT00085397	Vaccine therapy in treating patients with stage III or stage IV melanoma	MA10
NCT00085436	DC vaccine combined with IL-2 and IFNα-2a in treating patients with mRCC	NH3
NCT00085488	vaccine therapy in treating patients with stage III or stage IV melanoma	NH4
NCT00087984	RNA-loaded dendritic cell cancer vaccine	CA11, NY3, NC6, CANADA1
NCT00088985	Vaccine therapy, trastuzumab, and vinorelbine in treating women with locally recurrent or metastatic breast cancer	NC7
NCT00090896	CP-675,206 (CTLA4-Blocking monoclonal antibody) combined with dendritic cell vaccine therapy in treating patients with stage III or stage IV melanoma that cannot be removed with surgery	CA12
NCT00093522	Vaccine therapy with or without fludarabine in treating patients with stage IV kidney cancer	WI1
NCT00098917	Vaccine therapy in treating patients who are undergoing surgery for stage IB, Stage II, or stage IIIA non-small cell lung cancer	CA19
NCT00099593	Immunization against tumor cells in sezary syndrome	PA9
NCT00100971	Vaccine therapy in treating patients with acute myeloid leukemia	MA5

(continued)

Table 17.2 (continued)

Identifier	Title	State number
NCT00103116	Vaccine therapy in treating patients with stage I, stage II, or stage III non-small cell lung cancer	KY2
NCT00103142	Vaccine therapy in treating patients with liver or lung metastases from colorectal cancer	DC2, FL8, NC13, OR2, SC2
NCT00107159	Vaccine therapy in treating patients with unresected stage III or stage IV melanoma	CA25
NCT00107185	Vaccine therapy in treating young patients who are undergoing surgery for malignant glioma	CA28
NCT00107211	Vaccine therapy in treating patients who are undergoing surgery for ductal carcinoma in situ of the breast	PA20
NCT00108264	Tumor RNA transfected dendritic cell vaccines	NC3
NCT00124124	Comparison of dendritic cells versus montanide as adjuvants in a melanoma vaccine	NY10
NCT00125749	Vaccination of patients with stage IV melanoma with dendritic cells	TX11
NCT00126685	Vaccine therapy in treating patients with stage IV melanoma	GERMANY1
NCT00128622	Denileukin diftitox followed by vaccine therapy in treating patients with metastatic cancer	DC1, NC12
NCT00176761	Tumor-pulsed dendritic cells used as a tumor vaccine	MI4
NCT00186316	Vaccine therapy for multiple myeloma utilizing idiotype-pulsed allogeneic dendritic cells	CA26
NCT00197912	Dendritic cell based therapy of malignant melanoma	DENMARK1 DENMARK5
NCT00197925	Dendritic cell based therapy of metastatic breast cancer	DENMARK8
NCT00228189	Carcinoembryonic antigen-loaded dendritic cells in advanced colorectal cancer patients	NETHERLANDS3
NCT00243529	Peptide-pulsed versus RNA-transfected dendritic cell vaccines in melanoma patients	NETHERLANDS1
NCT00266110	Vaccine therapy, trastuzumab, and vinorelbine in treating patients with locally recurrent or metastatic breast cancer	NC8
NCT00289341	Safety and effectiveness of a vaccine for prostate cancer that uses each patients' own immune cells	NY7
NCT00309829	Vaccine therapy in treating patients with newly diagnosed stage IV kidney cancer	CA23
NCT00313235	Combined modality treatment for patients with stage IV melanoma	TX4
NCT00323115	Phase II feasibility study of dendritic cell vaccination for newly diagnosed glioblastoma multiforme	NH1

(continued)

Table 17.2 (continued)

Identifier	Title	State number
NCT00334776	Vaccine therapy in treating patients with metastatic melanoma	CA13, MI3
NCT00338377	Lymphodepletion plus adoptive cell transfer with or without dendritic cell immunization	TX7
NCT00345293	Dendritic cell vaccine study (DC/PC3) for prostate cancer	NY1
NCT00365872	External beam radiation with intratumoral injection of dendritic cells as neo-adjuvant treatment for sarcoma	FL9
NCT00374049	MUC1 vaccine in conjunction with Poly-ICLC in patients with recurrent and/or advanced prostate cancer	PA22
NCT00377247	Active immunization of patients with carcinoma of oral cavity or oropharynx with autologous dendritic cells transfected with DNA from autologous tumor	PA21
NCT00390338	Vaccine therapy in treating patients with stage III or stage IV melanoma	PA4
NCT00402142	Dendritic cell vaccine in HIV-1 infection	SPAIN1
NCT00404339	Vaccine therapy in treating patients with head and neck cancer	PA11
NCT00405327	A pilot study of tumor cell vaccine for high-risk solid tumor patients following stem cell transplantation	MI1
NCT00436930	Vaccine therapy and GM-CSF in treating patients with recurrent or metastatic melanoma	CA18
NCT00442754	Dendritic cells in lung cancer	DENMARK7
NCT00445913	Autologous dendritic cell therapy for type 1 diabetes suppression: a safety study	PA5
NCT00458536	Vaccination of patients with renal cell cancer with dendritic cell tumor fusions and GM-CSF	MA11
NCT00459069	The use of dendritic cell/tumor fusions as a novel tumor vaccine in patients with multiple myeloma	MA8
NCT00478452	Dendritic cell vaccine for high-risk ovarian cancer patients	PA6
NCT00492947	Dendritic cell vaccine for head and neck cancer	MD2
NCT00510133	A study of active immunotherapy with GRNVAC1 in patients with acute myelogenous leukemia (AML)	GA2, IL3, MO2, NE1, OH2, TX6
NCT00510497	Autologous dendritic cell vaccine in HIV1 infection	PA2
NCT00514189	Feasibility study of acute myelogenous leukemia mRNA plus lysate loaded dendritic cell vaccines	TX2

(continued)

Table 17.2 (continued)

Identifier	Title	State number
NCT00521287	Impaired immunity in patients with cancer: influence of cancer stage, chemotherapy, and cytomegalovirus infection	TAIWAN1
NCT00558051	Alpha-type-1 dendritic cell-based vaccines in patients with metastatic colorectal cancer	PA18
NCT00576446	Surgical resection with gliadel wafer followed by dendritic cells vaccination for malignant glioma patients	CA5
NCT00576537	Tumor lysate pulsed dendritic cell immunotherapy for patients with brain tumors	CA1
NCT00576641	Immunotherapy for patients with brain stem glioma and glioblastoma	CA6
NCT00601094	Vaccine therapy in treating patients with stage IIIB, stage IV, or recurrent non-small cell lung cancer	CA24
NCT00612001	Vaccine therapy in treating patients with malignant glioma	CA9
NCT00617409	To Immunize pts w extensive stage SCLC combined w chemo w or w/oall trans retinoic acid	FL4
NCT00622401	Vaccination of patients with breast cancer with dendritic cell/tumor fusions and IL-12	MA2
NCT00625755	A phase I/II study to assess the safety and efficacy of vaccinations with allogeneic dendritic cells: autologous tumor-derived cells subjected to electrofusions in patients with AJCC stage IV renal cell carcinoma	MA4
NCT00626483	Daclizumab in treating patients with newly diagnosed glioblastoma multiforme undergoing targeted immunotherapy and temozolomide-caused lymphopenia	NC5
NCT00626860	The use of dendritic cell/tumor hybridomas as a novel tumor vaccine in patients with advance melanoma	MA12
NCT00637117	Intratumoral dendritic cell vaccination combined with local radiotherapy in patients with recurrent lymphoma	TX14
NCT00639639	Vaccine therapy in treating patients with newly diagnosed glioblastoma multiforme	NC10
NCT00671554	Trial of melaxin cancer vaccine plus bacillus calmette-guerin (BCG) to treat malignant melanoma	SC5
NCT00672542	Immunotherapy of melanoma with tumor antigen rna and small inhibitory rna transfected autologous dendritic cells	NC15

(continued)

Table 17.2 (continued)

Identifier	Title	State number
NCT00683241	A phase I clinical trial of autologous dendritic cell vaccine for recurrent ovarian or primary peritoneal cancer	PA3
NCT00683670	Dendritic cells (white blood cells) vaccination for advanced melanoma	MO1
NCT00700167	Immune responses to antigen-bearing dendritic cells in patients with malignancy	NY4
NCT00703105	Ovarian dendritic cell vaccine trial	IL1
NCT00704938	Gene-modified lymphocytes, high-dose aldesleukin, and vaccine therapy in treating patients with progressive or recurrent metastatic cancer	MD7
NCT00715078	To evaluate sipuleucel-t manufactured with different concentrations of PA2024 antigen	CA38, DC6, IN7, NY18, OR7, WA11
NCT00715832	A phase I cancer vaccine study for patients with metastatic breast cancer	AL1
NCT00722098	Comparison study of dendritic cell vaccine with and without cyclophosphamide to treat stage IV melanoma patients	TX1
NCT00766753	Vaccination-dendritic cells with peptides for recurrent malignant gliomas	PA17
NCT00776295	Autologous SCT followed by dendritic cell p53 vaccination in patients with limited stage small cell lung cancer	FL5
NCT00779402	Provenge (TM) for the treatment of hormone sensitive prostate cancer; sipuleucel-T in metastatic castrate resistant prostate cancer (CRPC) patients previously treated on dendreon study P-11	CA15, CO1, CO5, IL2, NY5, NC9, OH3, OR1, PA10, TN2, VA1, WA3, WA8
NCT00796770	Vaccination of HIV-1 infected patients with dendritic cells in addition to antiretroviral treatment —(DALIA Trial)	TX5
NCT00798629	Adenovirus CCL-21 transduced MART-1/gp100/tyrosinase/NY-ESO-1 peptide-pulsed dendritic cells matured	FL6
NCT00799110	Vaccination of patients with ovarian cancer with dendritic cell/tumor fusions with granulocyte macrophage colony-stimulating factor (GM-CSF) and imiquimod	MA3
NCT00814892	Vaccine therapy in treating patients with non-metastatic prostate cancer	MN2
NCT00833781	A pilot study of a dendritic cell vaccine in HIV-1 infected subjects	MA1
NCT00834002	Dendritic cell vaccination for patients with acute myeloid leukemia in remission	BELGIUM2

(continued)

Table 17.2 (continued)

Identifier	Title	State number
NCT00856154	Vaccination with autologous dendritic cells pulsed with HIV-antigens for treatment of patients with chronic HIV-infection	DENMARK6
NCT00862303	DC vaccine therapy combined with cytokine-induced killer cell in treating patients with renal cell carcinoma	CHINA1
NCT00868114	Direct tumor injection KLH-pulsed dendritic cells in unresectable pancreatic cancer	NC1
NCT00879489	Cellular immunotherapy study with autologous dendritic cells loaded with oncofetal antigen/iLRP in patients with metastatic breast cancer	AL3
NCT00893945	Autologous dendritic cells pulsed with autologous apoptotic tumor cells administered to patients with brain tumors	NY11
NCT00901342	Open label study of sipuleucel-T	DC5, IL6, IN6, MD14, NY17, NC21, TN6, TX16, WA10, WI3
NCT00910650	Study of gene-modified immune cells in patients with advanced melanoma	CA16
NCT00913913	Bevacizumab, autologous tumor/DC vaccine, IL-2 and IFNα-2b in metastatic renal cell carcinoma (RCC) patients	NH2
NCT00923143	Vaccine therapy in treating patients with ductal carcinoma in situ of the breast	PA15
NCT00923351	Therapy to treat ewing's sarcoma, rhabdomyosarcoma or neuroblastoma	MD3
NCT00923910	Wilm's tumor 1 protein vaccine to treat cancers of the blood	MD4
NCT00935558	Dendritic cell based therapy for breast cancer patients	DENMARK3
NCT00937183	Dendritic cell vaccine in treating patients with indolent B cell lymphoma or multiple myeloma	POLAND1
NCT00940004	Toll-like receptor (TLR) ligand matured dendritic cell vaccination in melanoma patients	NETHERLANDS5
NCT00948480	Vaccine biotherapy of cancer: autologous tumor cells and dendritic cells	CA21
NCT00961844	Trial for vaccine therapy with dendritic cells in patients with metastatic malignant melanoma	NORWAY3
NCT00970203	Alpha-Type 1 dendritic Cell (DC)-based vaccines loaded with allogeneic prostate cell lines in combination with androgen ablation in patients with prostate cancer	PA1
NCT00978913	Transfected dendritic cell based therapy for patients with breast cancer or malignant melanoma	DENMARK2

(continued)

Table 17.2 (continued)

Identifier	Title	State number
NCT00988312	Dendritic Cells(DC)-based id vaccination in stage-I myeloma	GERMANY5
NCT01006044	Efficacy and safety of autologous dendritic cell vaccination in glioblastoma multiforme after complete surgical resection	SPAIN4
NCT01042366	Dendritic cells (DC) vaccine for metastatic melanoma	PA7
NCT01066390	A study on the safety and immunogenicity of combined intradermal and intravenous administration of an autologous mRNA electroporated dendritic cell vaccine in patients with previously treated unresectable stage III or IV melanoma	BELGIUM1
NCT01067287	Blockade of PD-1 in conjunction with the dendritic cell/myeloma vaccines following stem cell transplantation	MA7, ISRAEL1
NCT01068509	Ovarian cancer vaccine for patients in remission; ovarian cancer vaccine for patients who have progressed during the CAN-003 study; ovarian cancer vaccine for patients who have progressed during the CAN-003 study	FL1, GA1, IN1, NJ1, NY2, NC2, OH1, SC1, WA1, AUSTRALIA1, AUSTRALIA2
NCT01082198	Melanoma vaccine in treating patients with stage III melanoma after surgery to remove lymph nodes	POLAND2
NCT01096602	Blockade of PD-1 in conjunction with the dendritic Cell/AML vaccine following chemotherapy induced remission	MA9
NCT01132014	Autologous OC-DC vaccine in ovarian cancer	PA12
NCT01189383	IL15 dendritic cell vaccine for patients with resected stage IIIc and stage IV melanoma	TX3
NCT01197625	Vaccine therapy in curative resected prostate cancer patients	NORWAY2
NCT01204684	Dendritic cell vaccine for patients with brain tumors	CA10
NCT01216436	Local modulation of immune receptors to enhance the response to dendritic cell vaccination in metastatic melanoma	NC14
NCT01239875	Vaccine therapy with or without cryosurgery in treating patients with residual, relapsed, or refractory B cell non-hodgkin lymphoma	MN1
NCT01241162	Decitabine followed by a cancer antigen vaccine for patients with neuroblastoma and sarcoma	KY1
NCT01278914	Trial of vaccine therapy with mRNA- transfected dendritic cells in patients with androgen resistant metastatic prostate cancer	NORWAY4

(continued)

Table 17.2 (continued)

Identifier	Title	State number
NCT01302821	C11 AMT positron emission tomography (PET) imaging in patients with metastatic invasive breast cancer	FL3
NCT01312376	Autologous T-cells combined with autologous OC-DC vaccine in ovarian cancer	PA16
NCT01326104	Vaccine immunotherapy for recurrent medulloblastoma and primitive neuroectodermal tumor (PNET)	NC25
NCT01334047	Trial of vaccine therapy in recurrent platinum sensitive ovarian cancer patients	NORWAY1
NCT01338012	Sipuleucel-T in metastatic castrate resistant prostate cancer (CRPC) patients previously treated on dendreon study P-11 (NCT00779402)	CA37, OR6
NCT01339663	Vaccine therapy following therapeutic autologous lymphocytes and cyclophosphamide in treating patients with metastatic melanoma	WA5
NCT01348256	Study with dendritic cell immunotherapy in resected hepatic metastasis of colorectal carcinoma	SPAIN3
NCT01353222	DN24-02 as adjuvant therapy in subjects with high risk HER2+ urothelial carcinoma	AZ1, CO4, CA35, CT1, FL13, GA4, IL7, IN5, KS1, MA19, MD13, MI6, MN6, NE3, NJ5, NY16, NC20, OH8, OR5, PA25, TN5, WA9 SC8, TX19, VA2, WI4
NCT01413295	Randomized trial with dendritic cells in patients with metastatic colorectal cancer	SPAIN2
NCT01431391	Sequencing of sipuleucel-T and ADT in men with non-metastatic prostate cancer	AL2, CA41, CO7, MD16, NV1, NY20, SC7, TX18, WA13
NCT01441765	PD-1 alone or with dendritic cell/renal cell carcinoma fusion cell vaccine	MA6
NCT01446731	Dendritic cell vaccination and docetaxel for patients with prostate cancer	DENMARK4
NCT01456104	Immune responses to autologous langerhans-type dendritic cells electroporated with mrna encoding a tumor-associated antigen in patients with malignancy: a single-arm phase i trial in melanoma	NY8
NCT01472627	Effects of bortezomib-based therapy on cellular immunity and response to DC/myeloma fusion vaccines in vitro	MA21

(continued)

Table 17.2 (continued)

Identifier	Title	State number
NCT01483274	Decitabine and vaccine therapy for patients with relapsed AML following allogeneic stem cell transplantation	PA14
NCT01487863	Concurrent versus sequential treatment with sipuleucel-T and abiraterone in men with metastatic castrate resistant prostate cancer (mCRPC)	CO6, DC7, IN8, MD15, NE4, NY19, OR8, SC6, TN7, WA12
NCT01522820	*Vaccine therapy with or without sirolimus in treating patients with NY-ESO-1 expressing solid tumors*	*NY6*
NCT01530698	Single-step antigen loading and TLR activation of dendritic cells in melanoma patients	NETHERLANDS2
NCT01567202	Study of DC vaccination against glioblastoma	CHINA2
NCT01574222	A vaccine trial for patients with stage IIIB, IV, or recurrent non-small cell lung cancer	CA30
NCT01617629	Ovarian cancer vaccine for patients who have progressed during the CAN-003 study	CA7, FL2, FL2, IN2, WA2
NCT01635283	Vaccine for patients with newly diagnosed or recurrent low-grade glioma	CA8
NCT01671592	Safety of labeled dendritic cell (DC) vaccines and feasibility of tracking by magnetic resonance imaging (MRI)	PA13
NCT01686334	Efficacy study of dendritic cell vaccination in patients with acute myeloid leukemia in remission	BELGIUM3
NCT01690377	Plasmacytoid dendritic cell vaccines in metastatic melanoma patients	NETHERLANDS4
NCT01697527	Gene and vaccine therapy in treating patients with advanced malignancies	CA3
NCT01730118	Ad/HER2/Neu dendritic cell cancer vaccine testing	MD1
NCT00005947	Vaccine therapy in treating patients with metastatic prostate cancer that has not responded to hormone therapy	CA33
NCT00045968	Study of a Drug [DCVax®-L] to treat newly diagnosed GBM brain cancer	CA34
NCT00065442	Provenge® (Sipuleucel-T) active cellular immunotherapy treatment of metastatic prostate cancer after failing hormone therapy	CA32
NCT01068509	Ovarian cancer vaccine for patients in remission	CA2
NCT01487863	Concurrent versus sequential treatment with sipuleucel-T and abiraterone in men with metastatic castrate resistant prostate cancer (mCRPC)	CA39
NCT00766753	Vaccination-dendritic cells with peptides for recurrent malignant gliomas	PA17

(continued)

Table 17.2 (continued)

Identifier	Title	State number
NCT00776295	Autologous SCT followed by dendritic Cell p53 vaccination in patients with limited stage small cell lung cancer	FL5
NCT00779402	Provenge (TM) for the treatment of hormone sensitive prostate cancer; sipuleucel-T in metastatic castrate resistant prostate cancer (CRPC) patients previously treated on dendreon study P-11	CA15, CO1, CO5, IL2, NY5, NC9, OH3, OR1, PA10, TN2, VA1, WA3, WA8
NCT00796770	Vaccination of HIV-1 infected patients with dendritic cells in addition to antiretroviral treatment —(DALIA Trial)	TX5
NCT00798629	Adenovirus CCL-21 transduced MART-1/gp100/ tyrosinase/NY-ESO-1 peptide-pulsed dendritic cells matured	FL6
NCT00799110	Vaccination of patients with ovarian cancer with dendritic cell/tumor fusions with granulocyte macrophage colony-stimulating factor (GM-CSF) and imiquimod	MA3
NCT00814892	Vaccine therapy in treating patients with non-metastatic prostate cancer	MN2
NCT00833781	A pilot study of a dendritic cell vaccine in HIV-1 infected subjects	MA1
NCT00834002	Dendritic cell vaccination for patients with acute myeloid leukemia in remission	BELGIUM2
NCT00856154	Vaccination with autologous dendritic cells pulsed with hiv-antigens for treatment of patients with chronic HIV-Infection	DENMARK6
NCT00862303	DC vaccine therapy combined with cytokine-induced killer cell in treating patients with renal cell carcinoma	CHINA1
NCT00868114	Direct tumor injection KLH-pulsed dendritic cells in unresectable pancreatic cancer	NC1
NCT00879489	Cellular immunotherapy study with autologous dendritic cells loaded with oncofetal antigen/iLRP in patients with metastatic breast cancer	AL3
NCT00893945	Autologous dendritic cells pulsed with autologous apoptotic tumor cells administered to patients with brain tumors	NY11
NCT00901342	Open label study of sipuleucel-T	DC5, IL6, IN6, MD14, NY17, NC21, TN6, TX16, WA10, WI3
NCT00910650	Study of gene-modified immune cells in patients with advanced melanoma	CA16
NCT00913913	Bevacizumab, autologous tumor/DC vaccine, IL-2 and IFNα-2b in metastatic renal cell carcinoma (RCC) patients	NH2

(continued)

Table 17.2 (continued)

Identifier	Title	State number
NCT00923143	Vaccine therapy in treating patients with ductal carcinoma in situ of the breast	PA15
NCT00923351	Therapy to treat ewing's sarcoma, rhabdomyosarcoma or neuroblastoma	MD3
NCT00923910	Wilm's tumor 1 protein vaccine to treat cancers of the blood	MD4
NCT00935558	Dendritic cell based therapy for breast cancer patients	DENMARK3
NCT00937183	Dendritic cell vaccine in treating patients with indolent B cell lymphoma or multiple myeloma	POLAND1
NCT00940004	Toll-like receptor (TLR) ligand matured dendritic cell vaccination in melanoma patients	NETHERLANDS5
NCT00948480	Vaccine biotherapy of cancer: autologous tumor cells and dendritic cells	CA21
NCT00961844	Trial for vaccine therapy with dendritic cells in patients with metastatic malignant melanoma	NORWAY3
NCT00970203	Alpha-type 1 dendritic cell (DC)-Based vaccines loaded with allogeneic prostate cell lines in combination with androgen ablation in patients with prostate cancer	PA1
NCT00978913	Transfected dendritic cell based therapy for patients with breast cancer or malignant melanoma	DENMARK2
NCT00988312	Dendritic cells(DC)-based id vaccination in stage-I myeloma	GERMANY5
NCT01006044	Efficacy and safety of autologous dendritic cell vaccination in glioblastoma multiforme after complete surgical resection	SPAIN4
NCT01042366	Dendritic cells (DC) vaccine for metastatic melanoma	PA7
NCT01066390	A study on the safety and immunogenicity of combined intradermal and intravenous administration of an autologous mRNA electroporated dendritic cell vaccine in patients with previously treated unresectable stage III or IV melanoma	BELGIUM1
NCT01067287	Blockade of PD-1 in conjunction with the dendritic cell/myeloma vaccines following stem cell transplantation	MA7, ISRAEL1
NCT01068509	Ovarian cancer vaccine for patients in remission; ovarian cancer vaccine for patients who have progressed during the CAN-003 study; ovarian cancer vaccine for patients who have progressed during the CAN-003 study	FL1, GA1, IN1, NJ1, NY2, NC2, OH1, SC1, WA1, AUSTRALIA1, AUSTRALIA2

(continued)

Table 17.2 (continued)

Identifier	Title	State number
NCT01082198	Melanoma vaccine in treating patients with stage III melanoma after surgery to remove lymph nodes	POLAND2
NCT01096602	Blockade of PD-1 in conjunction with the dendritic cell/aml vaccine following chemotherapy induced remission	MA9
NCT01132014	Autologous OC-DC vaccine in ovarian cancer	PA12
NCT01189383	IL15 dendritic cell vaccine for patients with resected stage IIIc and stage IV melanoma	TX3
NCT01197625	Vaccine therapy in curative resected prostate cancer patients	NORWAY2
NCT01204684	Dendritic cell vaccine for patients with brain tumors	CA10
NCT01216436	Local modulation of immune receptors to enhance the response to dendritic cell vaccination in metastatic melanoma	NC14
NCT01239875	Vaccine therapy with or without cryosurgery in treating patients with residual, relapsed, or refractory B cell non-hodgkin lymphoma	MN1
NCT01241162	Decitabine followed by a cancer antigen vaccine for patients with neuroblastoma and sarcoma	KY1
NCT01278914	Trial of vaccine therapy with mRNA- transfected dendritic cells in patients with androgen resistant metastatic prostate cancer	NORWAY4
NCT01302821	C11 AMT positron emission tomography (PET) imaging in patients with metastatic invasive breast cancer	FL3
NCT01312376	Autologous T-cells combined with autologous OC-DC vaccine in ovarian cancer	PA16
NCT01326104	Vaccine immunotherapy for recurrent medullo-blastoma and primitive neuroectodermal tumor (PNET)	NC25
NCT01334047	Trial of vaccine therapy in recurrent platinum sensitive ovarian cancer patients	NORWAY1
NCT01338012	Sipuleucel-T in metastatic castrate resistant pros-tate cancer (CRPC) patients previously treated on dendreon study P-11 (NCT00779402)	CA37, OR6
NCT01339663	Vaccine therapy following therapeutic autologous lymphocytes and cyclophosphamide in treating patients with metastatic melanoma	WA5
NCT01348256	Study with dendritic cell immunotherapy in resected hepatic metastasis of colorectal carcinoma	SPAIN3

(continued)

Table 17.2 (continued)

Identifier	Title	State number
NCT01353222	DN24-02 as adjuvant therapy in subjects with high risk HER2+ Urothelial carcinoma	AZ1, CO4, CA35, CT1, FL13, GA4, IL7, IN5, KS1, MA19, MD13, MI6, MN6, NE3, NJ5, NY16, NC20, OH8, OR5, PA25, TN5, WA9 SC8, TX19, VA2, WI4
NCT01413295	Randomized trial with dendritic cells in patients with metastatic colorectal cancer	SPAIN2
NCT01431391	Sequencing of sipuleucel-T and ADT in men with non-metastatic prostate cancer	AL2, CA41, CO7, MD16, NV1, NY20, SC7, TX18, WA13
NCT01441765	PD-1 alone or with dendritic cell/renal cell carcinoma fusion cell vaccine	MA6
NCT01446731	Dendritic cell vaccination and docetaxel for patients with prostate cancer	DENMARK4
NCT01456104	Immune responses to autologous langerhans-type dendritic cells electroporated with mRNA encoding a tumor-associated antigen in patients with malignancy: a single-arm phase i trial in melanoma	NY8
NCT01472627	Effects of bortezomib-based therapy on cellular immunity and response to DC/myeloma fusion vaccines in vitro	MA21
NCT01483274	Decitabine and vaccine therapy for patients with relapsed AML following allogeneic stem cell transplantation	PA14
NCT01487863	concurrent versus sequential treatment with sipuleucel-T and abiraterone in men with metastatic castrate resistant prostate cancer (mCRPC)	CO6, DC7, IN8, MD15, NE4, NY19, OR8, SC6, TN7, WA12
NCT01522820	Vaccine therapy with or without sirolimus in treating patients with NY-ESO-1 expressing solid tumors	NY6
NCT01530698	Single-step antigen loading and TLR activation of dendritic cells in melanoma patients	NETHERLANDS2
NCT01567202	Study of DC vaccination against glioblastoma	CHINA2
NCT01574222	A vaccine trial for patients with stage IIIB, IV, or recurrent non-small cell lung cancer	CA30
NCT01617629	Ovarian cancer vaccine for patients who have progressed during the CAN-003 study	CA7, FL2, FL2, IN2, WA2
NCT01635283	Vaccine for patients with newly diagnosed or recurrent low-grade glioma	CA8

(continued)

Table 17.2 (continued)

Identifier	Title	State number
NCT01671592	Safety of labeled dendritic Cell (DC) vaccines and feasibility of tracking by magnetic resonance imaging (MRI)	PA13
NCT01686334	Efficacy study of dendritic cell vaccination in patients with acute myeloid leukemia in remission	BELGIUM3
NCT01690377	Plasmacytoid dendritic cell vaccines in metastatic melanoma patients	NETHERLANDS4
NCT01697527	Gene and vaccine therapy in treating patients with advanced malignancies	CA3
NCT01730118	Ad/HER2/Neu dendritic cell cancer vaccine testing	MD1
NCT00005947	Vaccine therapy in treating patients with metastatic prostate cancer that has not responded to hormone therapy	CA33
NCT00045968	Study of a Drug (DCVax®-L) to treat newly diagnosed GBM brain cancer	CA34
NCT00065442	Provenge® (Sipuleucel-T) active cellular immunotherapy treatment of metastatic prostate cancer after failing hormone therapy	CA32
NCT01068509	Ovarian cancer vaccine for patients in remission	CA2
NCT01487863	Concurrent versus sequential treatment with sipuleucel-T and abiraterone in men with metastatic castrate resistant prostate cancer (mCRPC)	CA39

Acknowledgment We thank the late Dr. Ralph M. Steinman and his past colleagues for their tremendous works on DC-targeting strategy, and Dr. Seongho Ryu from Soonchunhyang Institute of Medi-bio Sciences (SIMS) for a "human body image" in the Fig. 17.1, and J-A Han for assistance with review of clinical data in Table 17.2. This work was supported by funds from the National Institutes of Health (1 R21 AI082331-01, PI. Y. Do). This study was also supported by the National Research Foundation of Korea (NRF) (2013R1A1A2005545 & 2011-0020163) and UNIST research fund (No. 1.120020.01).

References

Akira S, Takeda K, Kaisho T (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2:675–680

Albert ML, Sauter B, Bhardwaj N (1998) Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature 392:86–89

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413:732–738

Allan RS, Smith CM, Belz GT, van Lint AL, Wakim LM, Heath WR, Carbone FR (2003) Epidermal viral immunity induced by CD8alpha + dendritic cells but not by Langerhans cells. Science 301:1925–1928

Altieri DC (2003) Validating survivin as a cancer therapeutic target. Nat Rev Cancer 3:46–54

Anderson GW Jr, Leary SE, Williamson ED, Titball RW, Welkos SL, Worsham PL, Friedlander AM (1996) Recombinant V antigen protects mice against pneumonic and bubonic plague caused by F1-capsule-positive and -negative strains of *Yersinia pestis*. Infect Immun 64:4580–4585

Andrews GP, Heath DG, Anderson GW Jr, Welkos SL, Friedlander AM (1996) Fraction 1 capsular antigen (F1) purification from Yersinia pestis CO92 and from an Escherichia coli recombinant strain and efficacy against lethal plague challenge. Infect Immun 64:2180–2187

Argani P, Iacobuzio-Donahue C, Ryu B, Rosty C, Goggins M, Wilentz RE, Murugesan SR, Leach SD, Jaffee E, Yeo CJ, Cameron JL, Kern SE, Hruban RH (2001) Mesothelin is overexpressed in the vast majority of ductal adenocarcinomas of the pancreas: identification of a new pancreatic cancer marker by serial analysis of gene expression (SAGE). Clin Cancer Res 7:3862–3868

Baca-Estrada ME, Foldvari MM, Snider MM, Harding KK, Kournikakis BB, Babiuk LA, Griebel PP (2000) Intranasal immunization with liposome-formulated Yersinia pestis vaccine enhances mucosal immune responses. Vaccine 18:2203–2211

Bachem A, Guttler S, Hartung E, Ebstein F, Schaefer M, Tannert A, Salama A, Movassaghi K, Opitz C, Mages HW, Henn V, Kloetzel PM, Gurka S, Kroczek RA (2010) Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. J Exp Med 207:1273–1281

Baldwin SL, Shaverdian N, Goto Y, Duthie MS, Raman VS, Evers T, Mompoint F, Vedvick TS, Bertholet S, Coler RN, Reed SG (2009) Enhanced humoral and Type 1 cellular immune responses with Fluzone adjuvanted with a synthetic TLR4 agonist formulated in an emulsion. Vaccine 27:5956–5963

Banchereau J, Schuler-Thurner B, Palucka AK, Schuler G (2001) Dendritic cells as vectors for therapy. Cell 106:271–274

Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392:245–252

Bates JH, Irvin CG (2003) Measuring lung function in mice: the phenotyping uncertainty principle. J Appl Physiol 94:1297–1306

Bendelac A, Medzhitov R (2002) Adjuvants of immunity: harnessing innate immunity to promote adaptive immunity. J Exp Med 195:F19–F23

Bera TK, Pastan I (2000) Mesothelin is not required for normal mouse development or reproduction. Mol Cell Biol 20:2902–2906

Berger TG, Feuerstein B, Strasser E, Hirsch U, Schreiner D, Schuler G, Schuler-Thurner B (2002) Large-scale generation of mature monocyte-derived dendritic cells for clinical application in cell factories. J Immunol Methods 268:131–140

Bevan MJ (2004) Helping the CD8(+) T-cell response. Nat Rev Immunol 4:595–602

Blanc-Brude OP, Mesri M, Wall NR, Plescia J, Dohi T, Altieri DC (2003) Therapeutic targeting of the survivin pathway in cancer: initiation of mitochondrial apoptosis and suppression of tumor-associated angiogenesis. Clin Cancer Res 9:2683–2692

Bonifaz L, Bonnyay D, Mahnke K, Rivera M, Nussenzweig MC, Steinman RM (2002) Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8 + T cell tolerance. J Exp Med 196:1627–1638

Bonifaz LC, Bonnyay DP, Charalambous A, Darguste DI, Fujii S, Soares H, Brimnes MK, Moltedo B, Moran TM, Steinman RM (2004) In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. J Exp Med 199:815–824

Bozzacco L, Trumpfheller C, Siegal FP, Mehandru S, Markowitz M, Carrington M, Nussenzweig MC, Piperno AG, Steinman RM (2007) DEC-205 receptor on dendritic cells mediates presentation of HIV gag protein to $CD8^+$ T cells in a spectrum of human MHC I haplotypes. Proc Natl Acad Sci USA 104:1289–1294

Brubaker RR (1991) The V antigen of yersiniae: an overview. Contrib Microbiol Immunol 12:127–133

Bubeck SS, Cantwell AM, Dube PH (2007) Delayed inflammatory response to primary pneumonic plague occurs in both outbred and inbred mice. Infect Immun 75:697–705

Caminschi I, Proietto AI, Ahmet F, Kitsoulis S, Shin Teh J, Lo JC, Rizzitelli A, Wu L, Vremec D, van Dommelen SL, Campbell IK, Maraskovsky E, Braley H, Davey GM, Mottram P, van de Velde N, Jensen K, Lew AM, Wright MD, Heath WR, Shortman K, Lahoud MH (2008) The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement. Blood 112:3264–3273

Caskey M, Lefebvre F, Filali-Mouhim A, Cameron MJ, Goulet JP, Haddad EK, Breton G, Trumpfheller C, Pollak S, Shimeliovich I, Duque-Alarcon A, Pan L, Nelkenbaum A, Salazar AM, Schlesinger SJ, Steinman RM, Sekaly RP (2011) Synthetic double-stranded RNA induces innate immune responses similar to a live viral vaccine in humans. J Exp Med 208:2357–2366

Chang K, Pastan I (1996) Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers. Proc Natl Acad Sci USA 93:136–140

Charalambous A, Oks M, Nchinda G, Yamazaki S, Steinman RM (2006) Dendritic cell targeting of survivin protein in a xenogeneic form elicits strong CD4+ T cell immunity to mouse survivin. J Immunol 177:8410–8421

Cheong C, Choi JH, Vitale L, He LZ, Trumpfheller C, Bozzacco L, Do Y, Nchinda G, Park SH, Dandamudi DB, Shrestha E, Pack M, Lee HW, Keler T, Steinman RM, Park CG (2010) Improved cellular and humoral immune responses in vivo following targeting of HIV Gag to dendritic cells within human anti-human DEC205 monoclonal antibody. Blood 116:3828–3838

Coler RN, Baldwin SL, Shaverdian N, Bertholet S, Reed SJ, Raman VS, Lu X, DeVos J, Hancock K, Katz JM, Vedvick TS, Duthie MS, Clegg CH, Van Hoeven N, Reed SG (2010) A synthetic adjuvant to enhance and expand immune responses to influenza vaccines. PLoS ONE 5:e13677

Corbeil LB, Gogolewski RP, Kacskovics I, Nielsen KH, Corbeil RR, Morrill JL, Greenwood R, Butler JE (1997) Bovine IgG2a antibodies to Haemophilus somnus and allotype expression. Can J Vet Res 61:207–213

Cyster JG (1999) Chemokines and the homing of dendritic cells to the T cell areas of lymphoid organs. J Exp Med 189:447–450

Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ, Hoff ST, Andersen P, Reed SG, Morris SL, Roederer M, Seder RA (2007) Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat Med 13:843–850

Davis KJ, Fritz DL, Pitt ML, Welkos SL, Worsham PL, Friedlander AM (1996) Pathology of experimental pneumonic plague produced by fraction 1-positive and fraction 1-negative *Yersinia pestis* in African green monkeys (*Cercopithecus aethiops*). Arch Pathol Lab Med 120:156–163

Dela Cruz JS, Lau SY, Ramirez EM, De Giovanni C, Forni G, Morrison SL, Penichet ML (2003) Protein vaccination with the HER2/neu extracellular domain plus anti-HER2/neu antibody-cytokine fusion proteins induces a protective anti-HER2/neu immune response in mice. Vaccine 21:1317–1326

den Haan JM, Lehar SM, Bevan MJ (2000) CD8(+) but not CD8(−) dendritic cells cross-prime cytotoxic T cells in vivo. J Exp Med 192:1685–1696

Disis ML, Calenoff E, McLaughlin G, Murphy AE, Chen W, Groner B, Jeschke M, Lydon N, McGlynn E, Livingston RB et al (1994) Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Cancer Res 54:16–20

Disis ML, Knutson KL, Schiffman K, Rinn K, McNeel DG (2000) Pre-existent immunity to the HER-2/neu oncogenic protein in patients with HER-2/neu overexpressing breast and ovarian cancer. Breast Cancer Res Treat 62:245–252

Do Y, Didierlaurent AM, Ryu S, Koh H, Park CG, Park S, Perlin DS, Powell BS, Steinman RM (2012) Induction of pulmonary mucosal immune responses with a protein vaccine targeted to the DEC-205/CD205 receptor. Vaccine 30:6359–6367

Do Y, Koh H, Park CG, Dudziak D, Seo P, Mehandru S, Choi JH, Cheong C, Park S, Perlin DS, Powell BS, Steinman RM (2010) Targeting of LcrV virulence protein from *Yersinia pestis* to dendritic cells protects mice against pneumonic plague. Eur J Immunol 40:2791–2796

Do Y, Park CG, Kang YS, Park SH, Lynch RM, Lee H, Powell BS, Steinman RM (2008) Broad T cell immunity to the LcrV virulence protein is induced by targeted delivery to DEC-205/CD205-positive mouse dendritic cells. Eur J Immunol 38:20–29

Du Y, Rosqvist R, Forsberg A (2002) Role of fraction 1 antigen of *Yersinia pestis* in inhibition of phagocytosis. Infect Immun 70:1453–1460

Dubensky TW Jr, Reed SG (2010) Adjuvants for cancer vaccines. Semin Immunol 22:155–161

Dudziak D, Kamphorst AO, Heidkamp GF, Buchholz VR, Trumpfheller C, Yamazaki S, Cheong C, Liu K, Lee HW, Park CG, Steinman RM, Nussenzweig MC (2007) Differential antigen processing by dendritic cell subsets in vivo. Science 315:107–111

Dzionek A, Sohma Y, Nagafune J, Cella M, Colonna M, Facchetti F, Gunther G, Johnston I, Lanzavecchia A, Nagasaka T, Okada T, Vermi W, Winkels G, Yamamoto T, Zysk M, Yamaguchi Y, Schmitz J (2001) BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. J Exp Med 194:1823–1834

Elvin SJ, Williamson ED (2004) Stat 4 but not Stat 6 mediated immune mechanisms are essential in protection against plague. Microb Pathog 37:177–184

Emini EA, Koff WC (2004) AIDS/HIV. Developing an AIDS vaccine: need, uncertainty, hope. Science 304:1913–1914

Engering A, Geijtenbeek TB, van Vliet SJ, Wijers M, van Liempt E, Demaurex N, Lanzavecchia A, Fransen J, Figdor CG, Piguet V, van Kooyk Y (2002) The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. J Immunol 168:2118–2126

Engering A, Pieters J (2001) Association of distinct tetraspanins with MHC class II molecules at different subcellular locations in human immature dendritic cells. Int Immunol 13:127–134

Eyles JE, Sharp GJ, Williamson ED, Spiers ID, Alpar HO (1998) Intra nasal administration of poly-lactic acid microsphere co-encapsulated *Yersinia pestis* subunits confers protection from pneumonic plague in the mouse. Vaccine 16:698–707

Eyles JE, Williamson ED, Spiers ID, Alpar HO (2000) Protection studies following broncho-pulmonary and intramuscular immunisation with yersinia pestis F1 and V subunit vaccines coencapsulated in biodegradable microspheres: a comparison of efficacy. Vaccine 18:3266–3271

Flynn BJ, Kastenmuller K, Wille-Reece U, Tomaras GD, Alam M, Lindsay RW, Salazar AM, Perdiguero B, Gomez CE, Wagner R, Esteban M, Park CG, Trumpfheller C, Keler T, Pantaleo G, Steinman RM, Seder R (2011) Immunization with HIV Gag targeted to dendritic cells followed by recombinant New York vaccinia virus induces robust T-cell immunity in nonhuman primates. Proc Natl Acad Sci USA 108:7131–7136

Fong L, Brockstedt D, Benike C, Wu L, Engleman EG (2001a) Dendritic cells injected via different routes induce immunity in cancer patients. J Immunol 166:4254–4259

Fong L, Hou Y, Rivas A, Benike C, Yuen A, Fisher GA, Davis MM, Engleman EG (2001b) Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy. Proc Natl Acad Sci U S A 98:8809–8814

Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM (2004) The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. J Exp Med 199:1607–1618

Gomez CE, Najera JL, Jimenez V, Bieler K, Wild J, Kostic L, Heidari S, Chen M, Frachette MJ, Pantaleo G, Wolf H, Liljestrom P, Wagner R, Esteban M (2007) Generation and immunogenicity of novel HIV/AIDS vaccine candidates targeting HIV-1 Env/Gag-Pol-Nef antigens of clade C. Vaccine 25:1969–1992

Granelli-Piperno A, Pritsker A, Pack M, Shimeliovich I, Arrighi JF, Park CG, Trumpfheller C, Piguet V, Moran TM, Steinman RM (2005) Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin/CD209 is abundant on macrophages in the normal human lymph node and is not required for dendritic cell stimulation of the mixed leukocyte reaction. J Immunol 175:4265–4273

Griffin KF, Conway BR, Alpar HO, Williamson ED (1998) Immune responses to V antigen of Yersinia pestis co-encapsulated with IFN-gamma: effect of dose and formulation. Vaccine 16:517–521

Grossman D, Kim PJ, Schechner JS, Altieri DC (2001) Inhibition of melanoma tumor growth in vivo by survivin targeting. Proc Natl Acad Sci USA 98:635–640

Guo M, Gong S, Maric S, Misulovin Z, Pack M, Mahnke K, Nussenzweig MC, Steinman RM (2000) A monoclonal antibody to the DEC-205 endocytosis receptor on human dendritic cells. Hum Immunol 61:729–738

Gurer C, Strowig T, Brilot F, Pack M, Trumpfheller C, Arrey F, Park CG, Steinman RM, Munz C (2008) Targeting the nuclear antigen 1 of Epstein-Barr virus to the human endocytic receptor DEC-205 stimulates protective T-cell responses. Blood 112:1231–1239

Harris NL, Watt V, Ronchese F, Le Gros G (2002) Differential T cell function and fate in lymph node and nonlymphoid tissues. J Exp Med 195:317–326

Hassan R, Ho M (2008) Mesothelin targeted cancer immunotherapy. Eur J Cancer 44:46–53

Hawiger D, Inaba K, Dorsett Y, Guo M, Mahnke K, Rivera M, Ravetch JV, Steinman RM, Nussenzweig MC (2001) Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J Exp Med 194:769–779

Heath DG, Anderson GW Jr, Mauro JM, Welkos SL, Andrews GP, Adamovicz J, Friedlander AM (1998) Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine. Vaccine 16:1131–1137

Heath WR, Belz GT, Behrens GM, Smith CM, Forehan SP, Parish IA, Davey GM, Wilson NS, Carbone FR, Villadangos JA (2004) Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. Immunol Rev 199:9–26

Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, Calderon B, Schraml BU, Unanue ER, Diamond MS, Schreiber RD, Murphy TL, Murphy KM (2008) Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. Science 322:1097–1100

Hill J, Eyles JE, Elvin SJ, Healey GD, Lukaszewski RA, Titball RW (2006) Administration of antibody to the lung protects mice against pneumonic plague. Infect Immun 74:3068–3070

Ho M, Bera TK, Willingham MC, Onda M, Hassan R, FitzGerald D, Pastan I (2007) Mesothelin expression in human lung cancer. Clin Cancer Res 13:1571–1575

Honko AN, Sriranganathan N, Lees CJ, Mizel SB (2006) Flagellin is an effective adjuvant for immunization against lethal respiratory challenge with *Yersinia pestis*. Infect Immun 74:1113–1120

Hsu FJ, Benike C, Fagnoni F, Liles TM, Czerwinski D, Taidi B, Engleman EG, Levy R (1996) Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. Nat Med 2:52–58

Hynes NE, Stern DF (1994) The biology of erbB-2/neu/HER-2 and its role in cancer. Biochim Biophys Acta 1198:165–184

Ichinohe T, Kawaguchi A, Tamura S, Takahashi H, Sawa H, Ninomiya A, Imai M, Itamura S, Odagiri T, Tashiro M, Chiba J, Sata T, Kurata T, Hasegawa H (2007) Intranasal immunization with H5N1 vaccine plus Poly I:Poly C12U, a Toll-like receptor agonist, protects mice against homologous and heterologous virus challenge. Microbes Infect 9:1333–1340

Ichinohe T, Watanabe I, Ito S, Fujii H, Moriyama M, Tamura S, Takahashi H, Sawa H, Chiba J, Kurata T, Sata T, Hasegawa H (2005) Synthetic double-stranded RNA poly(I:C) combined with mucosal vaccine protects against influenza virus infection. J Virol 79:2910–2919

Inglesby TV, Dennis DT, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Friedlander AM, Hauer J, Koerner JF, Layton M, McDade J, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Schoch-Spana M, Tonat K (2000) Plague as a biological weapon: medical and public health management. Working Group Civilian Biodefense. Jama 283:2281–2290

Islam A, Kageyama H, Takada N, Kawamoto T, Takayasu H, Isogai E, Ohira M, Hashizume K, Kobayashi H, Kaneko Y, Nakagawara A (2000) High expression of Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma. Oncogene 19:617–623

Iyoda T, Shimoyama S, Liu K, Omatsu Y, Akiyama Y, Maeda Y, Takahara K, Steinman RM, Inaba K (2002) The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. J Exp Med 195:1289–1302

Jiang W, Swiggard WJ, Heufler C, Peng M, Mirza A, Steinman RM, Nussenzweig MC (1995) The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. Nature 375:151–155

Jones T, Adamovicz JJ, Cyr SL, Bolt CR, Bellerose N, Pitt LM, Lowell GH, Burt DS (2006) Intranasal Protollin/F1-V vaccine elicits respiratory and serum antibody responses and protects mice against lethal aerosolized plague infection. Vaccine 24:1625–1632

Jongbloed SL, Kassianos AJ, McDonald KJ, Clark GJ, Ju X, Angel CE, Chen CJ, Dunbar PR, Wadley RB, Jeet V, Vulink AJ, Hart DN, Radford KJ (2010) Human CD141+ (BDCA-3) + dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. J Exp Med 207:1247–1260

Jonuleit H, Giesecke-Tuettenberg A, Tuting T, Thurner-Schuler B, Stuge TB, Paragnik L, Kandemir A, Lee PP, Schuler G, Knop J, Enk AH (2001) A comparison of two types of dendritic cell as adjuvants for the induction of melanoma-specific T-cell responses in humans following intranodal injection. Int J Cancer 93:243–251

Jung S, Unutmaz D, Wong P, Sano G, De los Santos K, Sparwasser T, Wu S, Vuthoori S, Ko K, Zavala F, Pamer EG, Littman DR, Lang RA (2002) In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. Immunity 17: 211–220

Kamath AT, Henri S, Battye F, Tough DF, Shortman K (2002) Developmental kinetics and lifespan of dendritic cells in mouse lymphoid organs. Blood 100:1734–1741

Kamath AT, Pooley J, O'Keeffe MA, Vremec D, Zhan Y, Lew AM, D'Amico A, Wu L, Tough DF, Shortman K (2000) The development, maturation, and turnover rate of mouse spleen dendritic cell populations. J Immunol 165:6762–6770

Kawai T, Akira S (2007) Antiviral signaling through pattern recognition receptors. J Biochem 141:137–145

Kim YS, Kim YJ, Lee JM, Han SH, Ko HJ, Park HJ, Pereboev A, Nguyen HH, Kang CY (2010) CD40-targeted recombinant adenovirus significantly enhances the efficacy of antitumor vaccines based on dendritic cells and B cells. Hum Gene Ther 21:1697–1706

Krown SE, Kerr D, Stewart WE 2nd, Field AK, Oettgen HF (1985) Phase I trials of poly(I, C) complexes in advanced cancer. J Biol Response Mod 4:640–649

Lampkin BC, Levine AS, Levy H, Krivit W, Hammond D (1985) Phase II trial of a complex polyriboinosinic-polyribocytidylic acid with poly-L-lysine and carboxymethyl cellulose in the treatment of children with acute leukemia and neuroblastoma: a report from the Children's Cancer Study Group. Cancer Res 45:5904–5909

Lathem WW, Crosby SD, Miller VL, Goldman WE (2005) Progression of primary pneumonic plague: a mouse model of infection, pathology, and bacterial transcriptional activity. Proc Natl Acad Sci USA 102:17786–17791

Lefrancois L, Puddington L (2006) Intestinal and pulmonary mucosal T cells: local heroes fight to maintain the status quo. Annu Rev Immunol 24:681–704

Levy HB, London W, Fuccillo DA, Baron S, Rice J (1976) Prophylactic control of simian hemorrhagic fever in monkeys by an interferon inducer, polyriboinosinic-polyribocytidylic acid-poly-L-lysine. J Infect Dis 133(Suppl):A256–A259

Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, Altieri DC (1998) Control of apoptosis and mitotic spindle checkpoint by survivin. Nature 396:580–584

Lichterfeld M, Kaufmann DE, Yu XG, Mui SK, Addo MM, Johnston MN, Cohen D, Robbins GK, Pae E, Alter G, Wurcel A, Stone D, Rosenberg ES, Walker BD, Altfeld M (2004) Loss of HIV-1-specific CD8+ T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific CD4+ T cells. J Exp Med 200:701–712

Lin JS, Kummer LW, Szaba FM, Smiley ST (2011a) IL-17 contributes to cell-mediated defense against pulmonary Yersinia pestis infection. J Immunol 186:1675–1684

Lin JS, Park S, Adamovicz JJ, Hill J, Bliska JB, Cote CK, Perlin DS, Amemiya K, Smiley ST (2010) TNFalpha and IFNgamma contribute to F1/LcrV-targeted immune defense in mouse models of fully virulent pneumonic plague. Vaccine 29:357–362

Lin JS, Szaba FM, Kummer LW, Chromy BA, Smiley ST (2011b) Yersinia pestis YopE contains a dominant CD8 T cell epitope that confers protection in a mouse model of pneumonic plague. J Immunol 187:897–904

Lin ML, Zhan Y, Proietto AI, Prato S, Wu L, Heath WR, Villadangos JA, Lew AM (2008) Selective suicide of cross-presenting CD8+ dendritic cells by cytochrome c injection shows functional heterogeneity within this subset. Proc Natl Acad Sci USA 105:3029–3034

Lindenstrom T, Agger EM, Korsholm KS, Darrah PA, Aagaard C, Seder RA, Rosenkrands I, Andersen P (2009) Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. J Immunol 182:8047–8055

Liu J, O'Brien KL, Lynch DM, Simmons NL, La Porte A, Riggs AM, Abbink P, Coffey RT, Grandpre LE, Seaman MS, Landucci G, Forthal DN, Montefiori DC, Carville A, Mansfield KG, Havenga MJ, Pau MG, Goudsmit J, Barouch DH (2009) Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. Nature 457:87–91

Longhi MP, Trumpfheller C, Idoyaga J, Caskey M, Matos I, Kluger C, Salazar AM, Colonna M, Steinman RM (2009) Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. J Exp Med 206:1589–1602

Mahnke K, Guo M, Lee S, Sepulveda H, Swain SL, Nussenzweig M, Steinman RM (2000) The dendritic cell receptor for endocytosis, DEC-205, can recycle and enhance antigen presentation via major histocompatibility complex class II-positive lysosomal compartments. J Cell Biol 151:673–684

Maldonado-Lopez R, De Smedt T, Michel P, Godfroid J, Pajak B, Heirman C, Thielemans K, Leo O, Urbain J, Moser M (1999) CD8alpha+ and CD8alpha- subclasses of dendritic cells direct the development of distinct T helper cells in vivo. J Exp Med 189:587–592

Marketon MM, DePaolo RW, DeBord KL, Jabri B, Schneewind O (2005) Plague bacteria target immune cells during infection. Science 309:1739–1741

Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC (2007) The vaccine adjuvant monophosphoryl lipid a as a TRIF-biased agonist of TLR4. Science 316:1628–1632

McIlroy D, Troadec C, Grassi F, Samri A, Barrou B, Autran B, Debre P, Feuillard J, Hosmalin A (2001) Investigation of human spleen dendritic cell phenotype and distribution reveals evidence of in vivo activation in a subset of organ donors. Blood 97:3470–3477

Medzhitov R (2001) Toll-like receptors and innate immunity. Nat Rev Immunol 1:135–145

Mellman I, Steinman RM (2001) Dendritic cells: specialized and regulated antigen processing machines. Cell 106:255–258

Mestas J, Hughes CC (2004) Of mice and not men: differences between mouse and human immunology. J Immunol 172:2731–2738

Meyer KF (1970) Effectiveness of live or killed plague vaccines in man. Bull World Health Organ 42:653–666

Meyer KF, Hightower JA, McCrumb FR (1974) Plague immunization. VI. Vaccination with the fraction i antigen of *Yersinia pestis*. J Infect Dis 129(Suppl):S41–S45

Mills KH, Ryan M, Ryan E, Mahon BP (1998) A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell-mediated immunity in protection against *Bordetella pertussis*. Infect Immun 66:594–602

Nakajima R, Brubaker RR (1993) Association between virulence of *Yersinia pestis* and suppression of gamma interferon and tumor necrosis factor alpha. Infect Immun 61:23–31

Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA, Vyas JM, Boes M, Ploegh HL, Fox JG, Littman DR, Reinecker HC (2005) CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science 307:254–258

Nordlund JJ, Wolff SM, Levy HB (1970) Inhibition of biologic activity of poly I: poly C by human plasma. Proc Soc Exp Biol Med 133:439–444

Novitsky V, Gilbert P, Peter T, McLane MF, Gaolekwe S, Rybak N, Thior I, Ndung'u T, Marlink R, Lee TH, Essex M (2003) Association between virus-specific T-cell responses and plasma viral load in human immunodeficiency virus type 1 subtype C infection. J Virol 77:882–890

Okada H, Bakal C, Shahinian A, Elia A, Wakeham A, Suh WK, Duncan GS, Ciofani M, Rottapel R, Zuniga-Pflucker JC, Mak TW (2004) Survivin loss in thymocytes triggers p53-mediated growth arrest and p53-independent cell death. J Exp Med 199:399–410

Overheim K, Lemoine S, Gallegos K, Fisher I, Monier A, Schneider C, Valderas M, Wilder J, Russell R, Sherwood R, Leclaire R (2012a) Increased dose of recombinant F1/V plague vaccine results in increased survival in plague-challenged cynomologus macaques. In: ASM biodefense and emerging diseases research meeting, Washington, DC

Overheim K, Lemoine S, Gallegos K, Fisher I, Monier A, Schneider C, Valderas M, Wilder J, Russell R, Sherwood R, Leclaire R (2012b) Increased protection of african green monkeys vaccinated with increasing doses of a recombinant F1/V plague vaccine. In: ASM Biodefense and Emerging Diseases research meeting, Washington, DC

Pack M, Trumpfheller C, Thomas D, Park CG, Granelli-Piperno A, Munz C, Steinman RM (2008) DEC-205/CD205+ dendritic cells are abundant in the white pulp of the human spleen, including the border region between the red and white pulp. Immunology 123:438–446

Palucka K, Banchereau J, Mellman I (2010) Designing vaccines based on biology of human dendritic cell subsets. Immunity 33:464–478

Pamer E, Cresswell P (1998) Mechanisms of MHC class I–restricted antigen processing. Annu Rev Immunol 16:323–358

Pantel A, Cheong C, Dandamudi D, Shrestha E, Mehandru S, Brane L, Ruane D, Teixeira A, Bozzacco L, Steinman RM, Longhi MP (2012) A new synthetic TLR4 agonist, GLA, allows dendritic cells targeted with antigen to elicit Th1 T-cell immunity in vivo. Eur J Immunol 42:101–109

Parent MA, Berggren KN, Kummer LW, Wilhelm LB, Szaba FM, Mullarky IK, Smiley ST (2005) Cell-mediated protection against pulmonary *Yersinia pestis* infection. Infect Immun 73:7304–7310

Parent MA, Wilhelm LB, Kummer LW, Szaba FM, Mullarky IK, Smiley ST (2006) Gamma interferon, tumor necrosis factor alpha, and nitric oxide synthase 2, key elements of cellular immunity, perform critical protective functions during humoral defense against lethal pulmonary Yersinia pestis infection. Infect Immun 74:3381–3386

Partidos CD, Hoebeke J, Moreau E, Chaloin O, Tunis M, Belliard G, Briand JP, Desgranges C, Muller S (2005) The binding affinity of double-stranded RNA motifs to HIV-1 Tat protein affects transactivation and the neutralizing capacity of anti-Tat antibodies elicited after intranasal immunization. Eur J Immunol 35:1521–1529

Perry RD, Fetherston JD (1997) *Yersinia pestis*–etiologic agent of plague. Clin Microbiol Rev 10:35–66

Pitt ML (2004) Animal models and correlates of protection for plague vaccines workshop, Gaithersburg, MD. http://www.fda.gov/cber/minutes/workshop-min.htm

Poulin LF, Salio M, Griessinger E, Anjos-Afonso F, Craciun L, Chen JL, Keller AM, Joffre O, Zelenay S, Nye E, Le Moine A, Faure F, Donckier V, Sancho D, Cerundolo V, Bonnet D, Reis e Sousa C (2010) Characterization of human DNGR-1+BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. J Exp Med 207:1261–1271

Powell BS, Andrews GP, Enama JT, Jendrek S, Bolt C, Worsham P, Pullen JK, Ribot W, Hines H, Smith L, Heath DG, Adamovicz JJ (2005) Design and testing for a nontagged F1-V fusion protein as vaccine antigen against bubonic and pneumonic plague. Biotechnol Prog 21:1490–1510

Pulendran B, Smith JL, Caspary G, Brasel K, Pettit D, Maraskovsky E, Maliszewski CR (1999) Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. Proc Natl Acad Sci USA 96:1036–1041

Ramamurthi KS, Schneewind O (2002) Type iii protein secretion in yersinia species. Annu Rev Cell Dev Biol 18:107–133

Randolph GJ, Angeli V, Swartz MA (2005) Dendritic-cell trafficking to lymph nodes through lymphatic vessels. Nat Rev Immunol 5:617–628

Reed DS, Martinez MJ (2006) Respiratory immunity is an important component of protection elicited by subunit vaccination against pneumonic plague. Vaccine 24:2283–2289

Regnault A, Lankar D, Lacabanne V, Rodriguez A, Thery C, Rescigno M, Saito T, Verbeek S, Bonnerot C, Ricciardi-Castagnoli P, Amigorena S (1999) Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. J Exp Med 189:371–380

Reis e Sousa C (2006) Dendritic cells in a mature age. Nat Rev Immunol 6:476–483

Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl JP, Ricciardi-Castagnoli P (2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol 2:361–367

Ridgway D (2003) The first 1000 dendritic cell vaccinees. Cancer Invest 21:873–886

Rosenberg ES, Billingsley JM, Caliendo AM, Boswell SL, Sax PE, Kalams SA, Walker BD (1997) Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. Science 278:1447–1450

Salazar AM, Levy HB, Ondra S, Kende M, Scherokman B, Brown D, Mena H, Martin N, Schwab K, Donovan D, Dougherty D, Pulliam M, Ippolito M, Graves M, Brown H, Ommaya A (1996) Long-term treatment of malignant gliomas with intramuscularly administered polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose: an open pilot study. Neurosurgery 38: 1096–1103 (Discussion 1103–1094)

Sallusto F, Cella M, Danieli C, Lanzavecchia A (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J Exp Med 182:389–400

Schmidt A, Rollinghoff M, Beuscher HU (1999) Suppression of TNF by V antigen of Yersinia spp. involves activated T cells. Eur J Immunol 29:1149–1157

Schnorrer P, Behrens GM, Wilson NS, Pooley JL, Smith CM, El-Sukkari D, Davey G, Kupresanin F, Li M, Maraskovsky E, Belz GT, Carbone FR, Shortman K, Heath WR, Villadangos JA (2006) The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. Proc Natl Acad Sci USA 103:10729–10734

Schuler-Thurner B, Schultz ES, Berger TG, Weinlich G, Ebner S, Woerl P, Bender A, Feuerstein B, Fritsch PO, Romani N, Schuler G (2002) Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. J Exp Med 195:1279–1288

Schuler G, Schuler-Thurner B, Steinman RM (2003) The use of dendritic cells in cancer immunotherapy. Curr Opin Immunol 15:138–147

Segura E, Albiston AL, Wicks IP, Chai SY, Villadangos JA (2009) Different cross-presentation pathways in steady-state and inflammatory dendritic cells. Proc Natl Acad Sci USA 106:20377–20381

Shortman K, Liu YJ (2002) Mouse and human dendritic cell subtypes. Nat Rev Immunol 2:151–161

Shortman K, Naik SH (2007) Steady-state and inflammatory dendritic-cell development. Nat Rev Immunol 7:19–30

Sing A, Rost D, Tvardovskaia N, Roggenkamp A, Wiedemann A, Kirschning CJ, Aepfelbacher M, Heesemann J (2002) Yersinia V-antigen exploits toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression. J Exp Med 196:1017–1024

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177–182

Sloots A, Mastini C, Rohrbach F, Weth R, Curcio C, Burkhardt U, Jager E, Forni G, Cavallo F, Wels WS (2008) DNA vaccines targeting tumor antigens to B7 molecules on antigen-presenting cells induce protective antitumor immunity and delay onset of HER-2/Neu-driven mammary carcinoma. Clin Cancer Res 14:6933–6943

Stahl-Hennig C, Eisenblatter M, Jasny E, Rzehak T, Tenner-Racz K, Trumpfheller C, Salazar AM, Uberla K, Nieto K, Kleinschmidt J, Schulte R, Gissmann L, Muller M, Sacher A, Racz P, Steinman RM, Uguccioni M, Ignatius R (2009) Synthetic double-stranded RNAs are adjuvants

for the induction of T helper 1 and humoral immune responses to human papillomavirus in rhesus macaques. PLoS Pathog 5:e1000373
Steinhagen F, Kinjo T, Bode C, Klinman DM (2011) TLR-based immune adjuvants. Vaccine 29:3341–3355
Steinman RM (2007) Dendritic cells: understanding immunogenicity. Eur J Immunol 37(Suppl 1): S53–S60
Steinman RM, Banchereau J (2007) Taking dendritic cells into medicine. Nature 449:419–426
Steinman RM, Dhodapkar M (2001) Active immunization against cancer with dendritic cells: the near future. Int J Cancer 94:459–473
Steinman RM, Pope M (2002) Exploiting dendritic cells to improve vaccine efficacy. J Clin Invest 109:1519–1526
Stephen EL, Sammons ML, Pannier WL, Baron S, Spertzel RO, Levy HB (1977) Effect of a nuclease-resistant derivative of polyriboinosinic-polyribocytidylic acid complex on yellow fever in rhesus monkeys (*Macaca mulatta*). J Infect Dis 136:122–126
Stevenson HC, Abrams PG, Schoenberger CS, Smalley RB, Herberman RB, Foon KA (1985) A phase I evaluation of poly(I, C)-LC in cancer patients. J Biol Response Mod 4:650–655
Tamm I, Wang Y, Sausville E, Scudiero DA, Vigna N, Oltersdorf T, Reed JC (1998) IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res 58:5315–5320
Taylor P, Gerder M, Moros Z, Feldmann M (1996) Humoral and cellular responses raised against the human HER2 oncoprotein are cross-reactive with the homologous product of the new proto-oncogene, but do not protect rats against B104 tumors expressing mutated neu. Cancer Immunol Immunother 42:179–184
Thomann JS, Heurtault B, Weidner S, Braye M, Beyrath J, Fournel S, Schuber F, Frisch B (2011) Antitumor activity of liposomal ErbB2/HER2 epitope peptide-based vaccine constructs incorporating TLR agonists and mannose receptor targeting. Biomaterials 32:4574–4583
Thomas AM, Santarsiero LM, Lutz ER, Armstrong TD, Chen YC, Huang LQ, Laheru DA, Goggins M, Hruban RH, Jaffee EM (2004) Mesothelin-specific CD8(+) T cell responses provide evidence of in vivo cross-priming by antigen-presenting cells in vaccinated pancreatic cancer patients. J Exp Med 200:297–306
Thompson KA, Strayer DR, Salvato PD, Thompson CE, Klimas N, Molavi A, Hamill AK, Zheng Z, Ventura D, Carter WA (1996) Results of a double-blind placebo-controlled study of the double-stranded RNA drug polyI:polyC12U in the treatment of HIV infection. Eur J Clin Microbiol Infect Dis 15:580–587
Trombetta ES, Mellman I (2005) Cell biology of antigen processing in vitro and in vivo. Annu Rev Immunol 23:975–1028
Trumpfheller C, Finke JS, Lopez CB, Moran TM, Moltedo B, Soares H, Huang Y, Schlesinger SJ, Park CG, Nussenzweig MC, Granelli-Piperno A, Steinman RM (2006) Intensified and protective CD4+ T cell immunity in mice with anti-dendritic cell HIV gag fusion antibody vaccine. J Exp Med 203:607–617
Trumpfheller C, Longhi MP, Caskey M, Idoyaga J, Bozzacco L, Keler T, Schlesinger SJ, Steinman RM (2012) Dendritic cell-targeted protein vaccines: a novel approach to induce T-cell immunity. J Intern Med 271:183–192
Trumpfheller C, Caskey M, Nchinda G, Longhi MP, Mizenina O, Huang Y, Schlesinger SJ, Colonna M, Steinman RM (2008) The microbial mimic polyIC induces durable and protective CD4+ T cell immunity together with a dendritic cell targeted vaccine. In: Proc Nat Acad Sci. USA (In Press)
Turley SJ, Inaba K, Garrett WS, Ebersold M, Unternaehrer J, Steinman RM, Mellman I (2000) Transport of peptide-MHC class II complexes in developing dendritic cells. Science 288:522–527
Valladeau J, Ravel O, Dezutter-Dambuyant C, Moore K, Kleijmeer M, Liu Y, Duvert-Frances V, Vincent C, Schmitt D, Davoust J, Caux C, Lebecque S, Saeland S (2000) Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. Immunity 12:71–81

Villadangos JA, Shortman K (2010) Found in translation: the human equivalent of mouse CD8 + dendritic cells. J Exp Med 207:1131–1134

Wang B, Kuroiwa JM, He LZ, Charalambous A, Keler T, Steinman RM (2009) The human cancer antigen mesothelin is more efficiently presented to the mouse immune system when targeted to the DEC-205/CD205 receptor on dendritic cells. Ann NY Acad Sci 1174:6–17

Wang B, Zaidi N, He LZ, Zhang L, Kuroiwa JM, Keler T, Steinman RM (2012) Targeting of the non-mutated tumor antigen HER2/neu to mature dendritic cells induces an integrated immune response that protects against breast cancer in mice. Breast Cancer Res 14:R39

Wei H, Wang S, Zhang D, Hou S, Qian W, Li B, Guo H, Kou G, He J, Wang H, Guo Y (2009) Targeted delivery of tumor antigens to activated dendritic cells via CD11c molecules induces potent antitumor immunity in mice. Clin Cancer Res 15:4612–4621

Williamson ED, Eley SM, Griffin KF, Green M, Russell P, Leary SE, Oyston PC, Easterbrook T, Reddin KM, Robinson A et al (1995) A new improved sub-unit vaccine for plague: the basis of protection. FEMS Immunol Med Microbiol 12:223–230

Witmer-Pack MD, Swiggard WJ, Mirza A, Inaba K, Steinman RM (1995) Tissue distribution of the DEC-205 protein that is detected by the monoclonal antibody NLDC-145. II. Expression in situ in lymphoid and nonlymphoid tissues. Cell Immunol 163:157–162

Zizzari IG, Veglia F, Taurino F, Rahimi H, Quaglino E, Belleudi F, Riccardo F, Antonilli M, Napoletano C, Bellati F, Benedetti-Panici P, Torrisi MR, Frati L, Nuti M, Rughetti A (2011) HER2-based recombinant immunogen to target DCs through FcgammaRs for cancer immunotherapy. J Mol Med (Berl) 89:1231–1240

Zuniga R, Lucchetti A, Galvan P, Sanchez S, Sanchez C, Hernandez A, Sanchez H, Frahm N, Linde CH, Hewitt HS, Hildebrand W, Altfeld M, Allen TM, Walker BD, Korber BT, Leitner T, Sanchez J, Brander C (2006) Relative dominance of Gag p24-specific cytotoxic T lymphocytes is associated with human immunodeficiency virus control. J Virol 80:3122–3125

Index

B.K. Nunnally et al. (eds.), *Vaccine Analysis: Strategies, Principles, and Control*,
DOI 10.1007/978-3-662-45024-6