Hildegund C. J. Ertl *Editor*

Rabies and Rabies Vaccines

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ISBN 978-3-030-21083-0 ISBN 978-3-030-21084-7 (eBook)
https://doi.org/10.1007/978-3-030-21084-7

This Springer imprint is published by the registered company Springer Nature Switzerland AG.
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

This book is dedicated to rabies virus and the different vaccines that are available or under investigation to combat this deadly pathogen. Why do we need another book on a disease that no one living in developed countries worries about unless it is time to go and get pets vaccinated. The fact we do not worry about rabies is the reason we decided to write this book. Rabies is a neglected disease, a disease we do not fear and therefore do not invest in. While I am writing this preface, we are in the middle of a dreadful pandemic caused by a new coronavirus, called SARS-CoV-2, which by now in mid-April of 2020 has killed over 100,000 people and is estimated to kill by August 2020 about 60,000 humans in the USA. Rabies each year kills 60,000 humans and that number is likely an underestimate. While SARS-CoV-2 has been dominating the news for weeks and resulted in massive global shutdowns, no one raises the alarm about rabies for it is a disease of the underprivileged. Rabies is preventable—we have vaccines and other biologicals that can protect humans, their pets, and even wildlife animals; nevertheless, the death toll due to this virus, which is the most fatal of all viruses that can infect humans, has not declined in decades. We hope that this book written by rabies experts, many of whom serve as advisors to international health agencies, such as the World Health Organization, will not only serve as a guidance for health care professionals dedicated to the treatment and prevention of rabies, but also raise awareness in others. I wish to thank the authors, who contributed.

Philadelphia, PA, USA — Hildegund C. J. Ertl

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Rabies Life Cycle, Transmission and Pathogenesis

Ashley C. Banyard and Anthony R. Fooks

Abstract

Rabies has shaped humanity for centuries and continues to be one of the few pathogens that have a near 100% case fatality rate following the onset of clinical disease. Rabies virus, as with all related viruses within the *Lyssavirus* genus, is spread via the mechanistic action of the bite of an infected animal. Lyssaviruses are strongly neurotropic and as such most often require the dermal barrier to be breached to enable access to and infection of the nervous system. The domestic dog represents the principal reservoir of rabies virus globally and 99% of human cases involve the bite of an infected dog. Lyssaviruses are predominantly shed through saliva and so although a bite appears to be the most efficient mechanism of transmission rare alternative routes, including organ transplantation, have been reported. Despite the near 100% case fatality rate, post-exposure intervention can prevent the development of clinical disease and resulting fatality. Vaccines against rabies have been available, in various forms, for over 100 years and alongside the observation that passive immunisation with rabies immunoglobulin can completely prevent disease when administered pre-clinically the disease is entirely preventable. However, in endemic regions the cost and availability of post-exposure vaccines and immunoglobulins often precludes their use and rabies develops with the concomitant high fatality reported in endemic countries. The need for efficacious and yet cheaper pan-lyssavirus vaccines and biologicals to both prevent and treat rabies remains an important issue for future development.

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H. C. J. Ertl (ed.), *Rabies and Rabies Vaccines*,
https://doi.org/10.1007/978-3-030-21084-7_1

Furthermore, a lack of adequate reporting systems means that rabies is grossly underreported and that the burden of disease may be considerably higher. This chapter considers the basis of rabies classification, epidemiology and pathogenesis is reviewed highlighting areas for potential improvement in our understanding of this important group of viruses.

Introduction

Rabies is an ancient zoonotic disease caused by viruses of the order Mononegavirales, family Rhabdoviridae, and genus *Lyssavirus* [1]. Rabies, or hydrophobia as it has been historically described through the profound fear of water observed in some clinical cases, is most commonly transmitted following the bite of an aggressive animal, most often a rabid dog. Rabies has shaped humanity for centuries with the first descriptions of rabies disease being recorded in the fourth century BC [2]. With a case fatality rate approaching 100% the disease, rabies, has shaped human history and remains one of the most globally feared pathogens. Whilst all members of the *Lyssavirus* genus are capable of causing rabies, only the prototype lyssavirus, rabies virus (RABV), is truly an important pathogen of humans and animals with an estimated 60,000 human fatalities being caused by rabies each year. The majority of human deaths occur across Africa and Asia where the virus is enzootic. Despite this death toll, both animal and human rabies are entirely preventable. In the 1800s, Louis Pasteur developed the first rabies vaccination during a time widely considered to be the dawn of vaccination. In the 100 years since the development of the first human vaccines, rabies vaccines have remained largely unchanged, with only the development of alternatives to the nerve tissue-derived vaccines through the establishment of vaccines prepared in cell culture, thereby altering the safety of the product available [3]. From these vaccines, the 0.5 IU/ml threshold, which was considered indicative for a protective virus neutralising antibody (VNA) response was established for human protection [4]. Despite the insensitivity of existing neutralisation tests to define a lower limit for VNA titres that could be considered as a protective level, this cut-off of 0.5 IU/ml has been universally accepted as a standard parameter that is representative of protective immunity against rabies virus [5]. Of course, the discovery of a further 15 proposed lyssaviruses dictates that a thorough assessment of neutralisation titre is required across the genus [6]. For the vaccination of dogs, several vaccine preparations are available although parenteral vaccination, though often logistically challenging, remains the most readily adopted mechanism of vaccination. Certainly, where responsible dog ownership is practised, parenteral vaccination and assessment of serological titre is undertaken [7]. In endemic areas, where free-roaming dogs are often abundant, options including oral vaccination have been proposed [8–11] although adoption of dog licencing and responsible ownership to reduce free-roaming populations is the only viable future option for rabies control in these areas [12].

Classification and Epidemiology of the Lyssaviruses

The prototype virus of the *Lyssavirus* genus is RABV. However, although RABV is the most notable cause of human fatalities from rabies, other lyssaviruses have been described that are capable of causing rabies and that have contributed to the human and animal death toll from these viruses. In total, there are 16 proposed members of the *Lyssavirus* genus with the majority of the lyssavirus species having been detected in bats [13]. Although the death toll associated with infection with lyssaviruses other than rabies is negligible, in RABV endemic regions the diagnostic tools required to type lyssaviruses species are generally lacking and as such the exact epidemiology of lyssaviruses is unclear [1]. Furthermore, whilst the existing rabies vaccines are able to stimulate a VNA response that protects against RABV, protection afforded against the other lyssaviruses is undefined and for specific lyssavirus species, it is acknowledged that current human vaccines are completely ineffective in preventing clinical disease and death [14, 15]. From an antigenic standpoint, this has led to the grouping of lyssaviruses into phylogroups, reflecting the data of vaccine protection within the *Lyssavirus* genus. All phylogroup I viruses are considered to be neutralised by the VNA response generated following rabies vaccination. For viruses in phylogroups II and III, either minimal or no protection is conferred by rabies vaccination. The taxonomic classification according to phylogenetic analysis of the nucleoprotein (N-gene) and detailing division of viral species into phylogroups is detailed in Fig. 1.

Whilst RABV, and the often horrific manifestations of the disease it causes, has shaped many elements of humanity [16], the remaining lyssaviruses are comparatively recent in their description [13]. Whilst the basic properties of the RABV life cycle and clinical disease are well defined, empirical data is still required to further our understanding of the lyssaviruses. Epidemiologically, RABV is present globally with all mammals being considered susceptible, although the development of clinical disease may depend on the infecting dose, the viral species involved and the host exposed [1]. The domestic dog, often free-roaming in areas where the virus is endemic [17], is the principal reservoir of the virus and almost all transmission events to humans involve the bite of an infected dog [5]. Other mechanisms of human infection have been reported that can deviate from the standard bite related exposure including rare events of organ transplantation [18] and interactions with bats where cryptic infection can occur through unknown exposures [19]. From an epidemiological standpoint, one confounding feature of lyssavirus epidemiology is the distribution of the different lyssavirus species globally. Rabies virus has been detected globally in terrestrial carnivores although infection of bats seems limited to bats in the Americas where cycles of infection exist in insectivorous, frugivorous and hematophagous bats [20]. In the Old World, whilst terrestrial carnivores maintain RABV, it has never been detected in bats there [21]. In contrast, the remaining 15 species of lyssaviruses have only been discovered in the Old World, with bat infection predominating. Such genetically divergent lyssaviruses include European bat lyssaviruses types 1 and 2, Bokeloh bat lyssavirus and Lleida bat lyssavirus within Europe; Aravan bat lyssavirus, Khujand bat lyssavirus, Irkut bat lyssavirus,

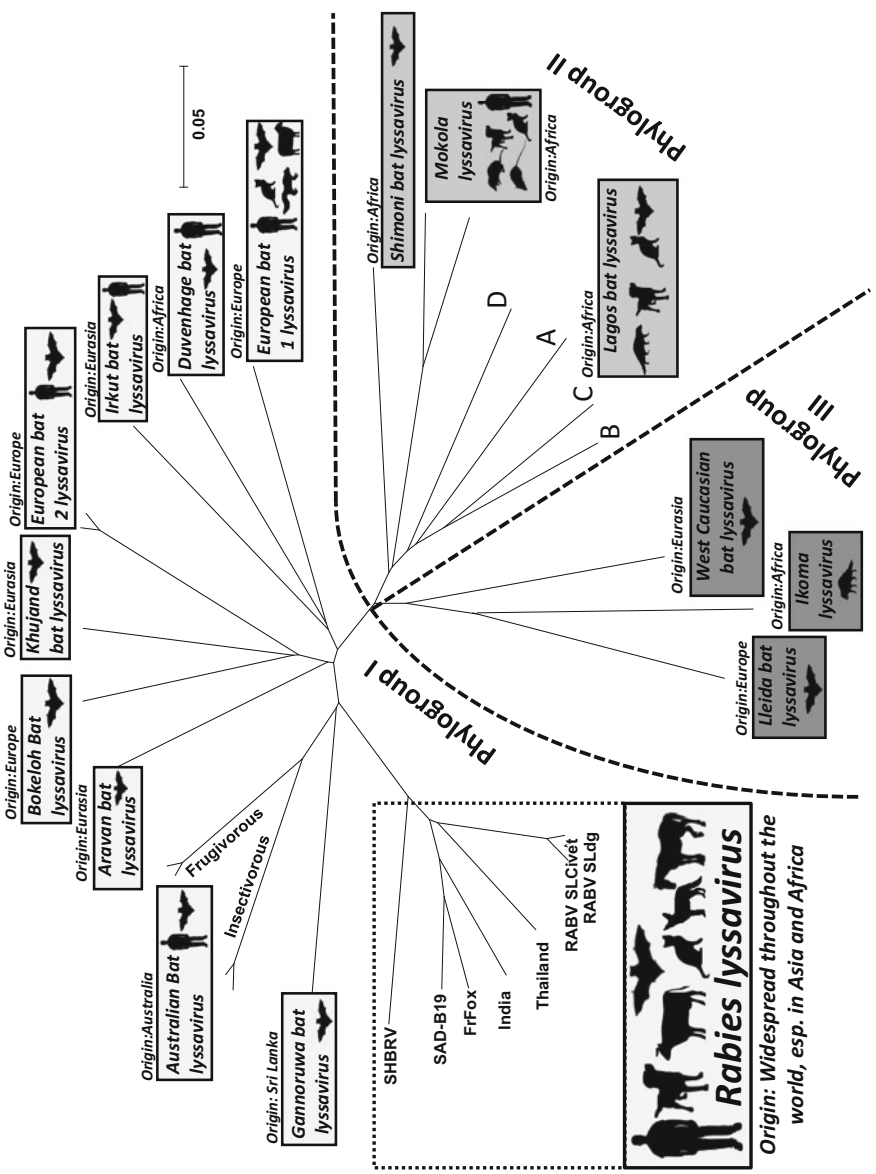

Fig. 1 Global members of the *Lyssavirus* genus

Gannoruwa bat lyssavirus and West Caucasian bat lyssavirus across Asia; Duvenhage bat lyssavirus, Shimoni bat lyssavirus, Ikoma lyssavirus, Mokola lyssavirus and Lagos bat lyssavirus across Africa; and Australian bat lyssavirus in Australasia. Interestingly, these lyssavirus species have never been detected in the Americas [22]. Other features of lyssavirus epidemiology, however, remain a paradox, particularly whether the origins of the 'cosmopolitan' strain of RABV and subsequent worldwide spread of this strain exist in the Old or New World.

Basic Viral Life Cycle

Lyssaviruses constitute a group of non-segmented negative-strand RNA viruses with small genomes of 11–12 kilobases encoding only five genes in the following conserved gene order: the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and Large polymerase protein (L). Conservation at the nucleotide level across the lyssavirus genome is high with maximum divergence of 60% being seen across the genome. Gene lengths and intergenic regions are also generally well conserved and the basic replication strategy of all non-segmented negative-strand viruses following a common theme as outlined below. Genome RNAs exist as fully encapsidated RNA molecules, protected from the harsh cytoplasmic milieu by the N-protein encapsidation. The negative-strand RNA genomes serve as the template for messenger RNAs that are generated by a transcriptase complex that includes the N, P and L proteins in association with the RNA. The complex of RNA, encapsidated in N and in association with P and L represents the minimal replicative unit for these viruses—the ribonucleoprotein complex (RNP). Viral transcription of mRNA species leads to the generation of viral proteins with mRNAs being translated on host cell ribosomes. An accumulation of viral proteins within the cell contributes to a switch from transcriptive to replicative activity whereby the polymerase generates full-length positive-sense genome strands of RNA that are co-transcriptionally encapsidated. These replicative intermediates then act as templates for the production of nascent genome negative-sense RNA that is encapsidated and released from the cell as nascent infectious virions.

Transmission and Pathogenesis

Lyssaviruses are most efficiently transmitted through exposure to virus-laden saliva that must cross the dermal barrier to infect although transmission can also occur through contact with mucous membranes. Once the virus has entered the host, it enters peripheral nerves and transports to neurons in the central nervous system (CNS) via retrograde axonal transport. Once in the CNS, the virus replicates, causing the development of clinical disease as it spreads through the brain. Behavioural changes are the result of this replication with two main outcomes of disease: furious and paralytic rabies. Whilst the late-stage disease in either outcome may differ, early stages of infection may be similar to paraesthesia and/or pruritus being evident at the

bite site. Further, incoordination, fever, and inhibitory spasms may be seen before the disease progresses to an aggressive or paralytic conclusion [23]. Cause of death is generally considered to be heart failure although replication within the brain can cause multi-organ system failure. For rabies, pathogenesis has been well defined in humans and animals although no symptoms are considered pathognomonic for rabies and as such only laboratory confirmation can prove a lyssavirus as the causative agent. In areas where diagnostic capabilities are unable to confirm the presence of a lyssavirus strain, infection may be considered to be caused by other infectious agents [24] or medical conditions [25]. For the remaining lyssaviruses, pathogenesis is poorly defined with only a small number of human cases being associated with infection [13]. Of those, the infection with European and Australian bat lyssaviruses has most extensively documented as they have occurred in countries with adequate health care and diagnostic capabilities to enable assessment of the infected individuals throughout the course of disease [26]. As seen with rabies, a progressive encephalitis was observed that resulted in death [27–29]. Following such high profile cases in humans in rabies-free areas, several recommendations were made regarding interactions with bats including a recommendation for pre-immunisation and use of personal protective equipment when handling any bats [30]. Where human infection has not been reported, and the association of a lyssavirus species is restricted to bat species, the epidemiology of these lyssaviruses is poorly defined. Certainly, numerous lyssaviruses exist as only single isolates and as such, there is limited information regarding their evolution and global distribution. Based on the genetic heterogeneity within the Old World lyssavirus species, it has been speculated that the evolutionary origins of lyssaviruses originated in the Paleo-Arctic regions [31], a hypothesis in direct contrast to the 'out of Africa' postulated previously [32]. This new hypothesis has led to the suggestion that the New World RABV evolved from a recent common ancestor in the Old World, probably in a sympatric bat host, before host switching from chiropteran into mammalian species. It is clear that the continued discovery of novel, phenotypically and genetically divergent lyssaviruses from different regions of the world prevents meaningful assessment of lyssavirus evolution, especially in instances where the number of isolates detected remains low.

Rabies Diagnosis

In endemic regions, the diagnosis of rabies is challenging due to a general lack of diagnostic capabilities and cultural practises that resist the submission of diagnostic material at post-mortem. The optimal sample for rabies diagnosis is brain material that can only be taken at post-mortem although options for ante-mortem testing, including saliva swabs and nuchal skin biopsies have shown utility [33]. For human rabies diagnosis, the requirement for brain sampling is problematic as post-mortem of the deceased is often culturally unacceptable in endemic regions and as such a definitive diagnosis is rarely made [34]. As previously described, this can lead to the misdiagnosis of rabies [24]. To this end, a high priority in the fight to eliminate rabies

is the development of diagnostic infrastructure at both animal and human health centres such that endemic regions have the capability to accurately diagnose the infection at both ante- and post-mortem using internationally validated diagnostic tools. The technological evolution of molecular techniques and next-generation sequencing has revolutionized the ability to diagnose infectious organisms although such technology is often lacking in resource-limited areas [35]. Future efforts should focus on enabling diagnosticians within endemic regions through the provision of tools and training to be able to accurately diagnose lyssavirus infections. At its simplest, local diagnosis can be confirmed at national or international reference laboratories [35].

Global Efforts to Eliminate Dog-Mediated Rabies

In a vital first step in the planned elimination of dog-mediated rabies, the World Health Organisation (WHO) and World Organisation for Animal Health (OIE) have mandated that human and animal rabies should be considered notifiable diseases. As a first stage, a system for diagnosis and surveillance should be initiated to ensure accurate reporting and notification of animal and human rabies cases [35]. However, despite this important definition, endemic areas often lack the laboratory infrastructure to enable timely reporting of cases. To this end, governments in endemic areas should follow a step-wise approach towards dog rabies elimination that links achievable goals on a structured pathway to disease elimination [12].

The WHO, OIE and the Food and Agriculture Organisation (FAO) have advocated the elimination of dog-mediated human rabies by 2030. This aim forms part of the Millennium Development Goals to reduce poverty and preventable childhood deaths from infectious diseases in resource-limited regions of the world and, although it is considered a neglected tropical disease, the burden on human life and cost of post-exposure prophylactic resources places rabies as a high priority [36]. The FAO in collaboration with the OIE and WHO has further developed a Progressive Control Pathway towards rabies elimination, in which the final stage is maintaining freedom from rabies in both human and animal populations [37]. As the vast majority of human cases of rabies arise following the bite from an infected dog, this aim, if successful, will all but eradicate terrestrial rabies, as wildlife reservoirs of the disease are thought to contribute to a fraction of human cases. However, improved rabies surveillance in the light of elimination of dog-mediated rabies might demonstrate that certain sylvatic reservoirs are of greater importance than others and that other lyssaviruses also pose a serious threat to human and animal health. The successful elimination of rabies from domestic species across Western Europe and North America has demonstrated the impact of elimination of the disease. However, the continued presence of sylvatic rabies in North America has highlighted that improvements to vaccination strategies for wildlife rabies are required to eliminate this remaining threat. It is unclear how much this situation will be seen across endemic areas in Africa and Asia, where free-roaming dog populations act as the principal reservoir and wildlife cases are rarely reported.

However, it is likely that similar factors will affect rabies elimination strategies in the longer term. Whilst the elimination of terrestrial rabies is an achievable goal with the concomitant reduction of human rabies cases worldwide, options to eliminate lyssaviruses in bat populations remain challenging. Consequently, the true eradication of lyssaviruses from all mammalian reservoirs, with comparison to the eradication of Smallpox and Rinderpest, is thought to be unachievable at present.

Clearly, the factors associated with the elimination of rabies include complex obstacles that require attention. The elimination of RABV from domestic dogs will drive a considerable reduction in human disease, but relies on the development of both human and veterinary infrastructure that will support a consistent and sustained programme of elimination [38]. To achieve the global elimination of dog-mediated rabies, it is estimated that investment of over US$6.3 billion is required [39]. Frustratingly, cultural and behavioural shifts in the management of dog populations would readily remove this obstacle to elimination. Seventy percent vaccine coverage has been defined as the cut-off for which vaccination can disrupt the transmission chain of the virus in free-roaming dog communities [40–42], and as such responsible dog ownership and vaccination is a simple mechanism that would enable the prevention of human rabies [43]. Where extensive free-roaming dog populations circulate, the oral vaccination of free-roaming dog populations has been proposed as a complementary measure to parenteral vaccination, but the delivery of vaccines in this way, in a manner that negates the risk of human consumption is challenging, as is any post-vaccinal assessment of immune responses [44]. Whilst the elimination of sylvatic rabies from Western Europe has demonstrated the utility of oral vaccination programmes, the challenges in achieving sufficient mastication of the vaccine in dogs to enable adequate exposure and seroconversion remains a surmountable obstacle [45]. In an era where dog-mediated rabies has been eliminated, the full impact of other lyssaviruses may be further defined and as such options for optimised, cross-reactive pan-lyssavirus vaccines that are affordable for use in economically restricted countries may need to be developed [6] although they are unlikely to be of interest unless a substantial threat from other lyssaviruses in causing human rabies cases is demonstrated [38].

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History of Rabies and Rabies Vaccines

Thirumeni Nagarajan and Charles E. Rupprecht

Abstract

Lyssaviruses continue to evolve and pose threats to humans, domestic animals, and wildlife. As a fatal disease of zoonotic importance, rabies is fortunately preventable, thanks to the advent of potent biologics. Pioneering works done during the last two centuries act as cornerstone of research making rabies diagnosis, prevention, control, and selective elimination possible. The field has benefited immensely from the tremendous scientific advancement during the last three decades in virology, molecular biology, vaccinology, and delivery systems. Vaccines have evolved from the first generation of crude nerve tissue-based products to recombinant vaccines. Cell culture-based inactivated rabies vaccines for intramuscular (IM) and intradermal (ID) use in humans continue to play a pivotal role when it comes to rabies prophylaxis. Parenteral and oral vaccinations prove time and again promising tools for rabies control in domestic animals and wildlife. Improvements likely to cause paradigm shift include products based on virus-like particles, obviating the need for a high containment facility; sparing usage of antigens using new adjuvants; delivery using novel systems; and direct inoculation of potent vaccines without the need of rabies immune globulin (RIG).

History of Rabies

No one knows how rabies first began. However, the enhanced sophistication of next-generation sequencing and metagenomics techniques has the capacity to identify novel and historical agents through genomic and transcriptomic databases. Related

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H. C. J. Ertl (ed.), *Rabies and Rabies Vaccines*,
https://doi.org/10.1007/978-3-030-21084-7_2

insights from paleovirology, concentrating upon viral remnants that integrated into host genomes long ago, may eventually allow a better glimpse of such potential origins [1–3]. Increasingly, endogenous viral elements (EVEs) offer faint footprints of ancient lineages that may have relatedness to contemporary taxa. For example, recent scanning and identification of rhabdovirus EVEs among several invertebrate species suggest that such endogenization has been operative in multiple crustaceans over millions of years [4]. Hence, rhabdovirus ancestors may have diverged correspondingly during the evolution of invertebrates, plants, and vertebrates, notably among the mammals. Could rabies have existed even before the origin of mammals? Why not, if one considers ideal characteristics for a successful rabies reservoir: a well-developed central nervous system (CNS); homeothermy; gregariousness; sharp, penetrating teeth; functional salivary glands; vagility; ideal distribution, and abundance; etc. By inference, multiple extant and extinct taxa possess such traits. As such, did certain dinosaur species contract rabies? Possibly, as it may be otherwise difficult to fathom proto-lyssaviruses passing up such a great abundance of biomass and clearly, why else would they be considered such "terrible lizards"?! Nevertheless, when and where lyssaviruses emerged among groups such as the Chiroptera remain controversial [5, 6].

Apart from such speculation, more recently, from the dawn of civilization, over the past 4000 years, a disease akin to rabies has been described in nearly every culture, among poets and philosophers alike. Scholars from ancient Babylon, China, India, Persia, Greece, and Rome all wrote about a syndrome associated with illness and animal bite. Likely, rabies began its close association with civilized society from the time of canine domestication and the organization of urban centers. Notwithstanding the presence of rabies in wildlife (and a complete lack of appreciation of the primary role of bats until the twentieth century), which must have predated disease occurrence in domestic animals, reports of rabid bears, foxes, jackals, wolves, etc., were much less commonly perceived in the Old World than in the pariah dog. Hence, much of the focus of this early writing had to do with natural history and associated clinical signs. By the Middle Ages, a bit of a nuanced approach evolved from mere observation to the prescription of remedies for the exposed person. These various treatments involved a cornucopia of spells, incantations, potions, amulets, mad stones, herbs, purgatives, odd diets, sea dunking, dancing, bleeding, transfusions, boiling oil, insect stings, chilli peppers, amputation, cauterization, etc. (some of which persist to the present day). Although rife with superstitions and incorrect assessments over the centuries, several pre-Pasteurian personalities added more introspection gradually, as science eventually held more sway than mere dogmatic repeats of "learned" expert opinion (Table 1).

Classical virology during the twentieth century involved an etiological and pathological focus initially upon filtration, animal isolation and adaptation, microscopic identification of non-specific (e.g., perivascular cuffing) and specific (e.g., Negri bodies) lesions in the CNS, applied biochemistry and electron microscopy up to the 1950s. Thereafter, diagnostic, antigenic, and genetic breakthroughs provided a substantive underpinning to the primary development of improved vaccines (Table 2). Clearly, given thousands of years of dreaded notoriety, as one of the

Table 1 Selected pre-twentieth century personalities and their contributions

Individual	Period	Notoriety
Aulus Cornelius Celsus	~25 AD	Roman author of *De Medicina* and an early proponent of wound treatment after bite
Pliny the Elder	~70 AD	Roman naturalist with attribution in the influence of temperatures on disease and believed dogs were most susceptible to rabies during the hottest seasons of the year, as well as the alleged importance of "tongue worms"
Galen of Pergamon	~200 AD	Greek physician who advised prompt local treatment and that bite wounds be kept open to avoid viral absorption
Ibn Sina (Avicenna)	~1000 AD	Persian physician who wrote a famous Canon of Medicine
Moses Maimonides	~1198	Talmudic scholar and author of a treatise on *Poisons and Their Antidotes*, who described long incubation periods in bitten persons
Girolamo Fracastoro	~1546	Italian physician who recognized a clear material basis of contagion for rabies infections
Giovanni Battista Morgani	~1769	Italian anatomist and author of *On the Seats and Causes of Disease*, who established a fundamental pathological principle that diseases such as rabies are not dispersed vaguely throughout the body, but originate locally, in specific organs and tissues, such as the nerves
Georg Gottfried Zinke	~1804	German investigator who demonstrated that virus could be transmitted by infectious saliva
Apollinaire Bouchardat	~1852	French pharmacist who was one of the first to speculate on the potential utility of inoculations against rabies
Pierre-Victor Galtier	~1881	French veterinarian who showed pathogen transmission via injection and bite, used rabbits as a research model, developed a concept for an early experimental intravenous vaccine producing immunity in sheep and who had a major influence upon Pasteur's later work

oldest recognized infectious diseases, a proper history of rabies is well beyond the scope of this chapter and the interested reader is referred to other relevant sources on this particular topic [e.g., 22–26].

History of Rabies Vaccines

Introduction

Rabies is a preventable yet fatal disease that is responsible for more than 60,000 deaths each year [27]. However, widespread underreporting of cases means that the actual number of deaths is likely to be higher. Poor and rural populations are disproportionately affected, with the majority of deaths occurring in children 15 years or younger in many parts of Asia and Africa [28]. Ninety-nine percent of human rabies cases result from dog bites and once symptoms begin, the disease is

Table 2 Notable twentieth century accomplishments augmenting vaccine development

Facet	Applied utility	Example
Ultrafiltration	Proof that the agent causing rabies could pass through filters that confined conventional bacteria	[7]
Histopathological stains	Detection of neuronal intracytoplasmic inclusion bodies as an anatomic–pathologic application for diagnosis using CNS tissue	[8]
Inactivation methods	Post-Pasteurian preservation and stabilization of nerve tissue-based vaccines via the use of carbolic acid, formalin, ultraviolet light, etc.	[9]
Neutralization protocols	Use of laboratory animals and eventually cell cultures for use in functional definition of antibody induction	[10]
Animal potency tests	Allowed measurement of the comparative strength of different vaccine lots to standards for licensing	[11]
Electron microscopy	Structural–functional insights to viral replication, transcription, and translation	[12]
Adaptation of virus propagation from adult species to an immature murine system	Suckling mouse brain production as an improvement over adult nervous tissue substrates	[13]
Advent of tissue culture	Provided an in vitro environment for viral propagation	[14]
Fluorescent antibody diagnosis	Ability to detect viral antigens during vaccine production	[15]
Protein purification	Indication that the outer glycoprotein was the only major contributing antigen to the production of virus-neutralizing antibodies	[16]
Monoclonal antibodies	Selection of safer pathogenic variants for modified live products	[17]
Molecular cloning	Bacterial plasmids used for gene expression and amino acid sequence deduction	[18]
RT-PCR	Amplification methods to discern primary viral products versus adventitious agents and contaminants	[19]
Genetic sequencing	Genomic characterization and precise identity of vaccinal seed strains	[20]
Reverse genetics	Technology for both pathobiology and vaccine application	[21]

almost invariably fatal [29]. Rabies is one of the few infectious diseases, which can be prevented through vaccination even after exposure. Vaccination is central to prevent fatality and represents the most effective strategy. Indeed, rabies vaccines have had a huge impact on health for more than a century by preventing deaths. They are indicated for both pre-exposure prophylaxis (PrEP) and postexposure prophylaxis (PEP) to prevent human rabies. The traditional PrEP with three doses of

vaccine given for a primary course is recommended for persons in high-risk groups such as veterinarians and their staff, animal handlers, rabies researchers, and certain laboratory workers. It should also be considered in persons (e.g., international travelers) who are likely to come in contact with rabid animals in areas or countries where dog or other animal rabies is enzootic and immediate access to appropriate medical care, including rabies vaccine and RIG, might be limited [30, 31]. Vaccination involves giving a series of intramuscular (IM) or intradermal (ID) injections of rabies vaccine to prime the immune system. This enables rapid recall of memory immune responses once a person is re-exposed to the rabies virus (RABV). Moreover, people who have received PrEP require fewer doses of rabies vaccine post-exposure and can be treated without RIG, which is very costly and often difficult to procure [32]. Ideal PEP involves prompt and thorough wound cleansing followed by passive immunization with RIG derived from either human (HRIG) or equine (ERIG) donors and vaccination typically with four doses of rabies vaccine (given in a series separated by several days) for persons previously unvaccinated. Persons who previously received a complete vaccination series (PrEP or PEP) should receive only two doses of vaccine (but even this number may be overkill in healthy subjects where a single dose may suffice).

Commercial Vaccines

Parenteral Vaccines

Nerve Tissue Vaccines: The First Generation

Louis Pasteur made history during the late 1880s by developing the first rabies vaccine, which was based on the central nervous tissue of rabbit origin and RABV inactivation involved a physical method (drying). This method had the risk of residual live virus and severe allergic reactions due to the presence of nervous tissue and myelin basic protein. However, this paved the way for the development of other nerve tissue vaccines (NTV) derived from adult sheep (Semple vaccine) by Sir David Semple at Central Research Institute (CRI), Kasauli, India [33]. Its manufacture involved propagation of RABV in adult sheep brain followed by inactivation using a chemical agent (phenol). It was widely used in several countries despite many issues associated with its use such as poor potency, need for administration of multiple doses and severe reactions including Guillain–Barre Syndrome (GBS) due to the presence of myelin [34]. It also suffered from the theoretical risk of transmission of Transmissible Spongiform Encephalopathies (TSE) including Scrapie [35]. All these drawbacks made the WHO to continuously discourage its use, which resulted in its eventual discontinuation in almost all countries. The quest for an NTV, which is relatively less reactogenic than Semple vaccine, resulted in the development of a suckling mouse brain (SMB) vaccine [36]. Its manufacture involved propagation of RABV in suckling mouse brain followed by its inactivation using phenol and partial purification. Its use in multiple countries for decades came to an end when most national regulatory authorities decided to discontinue its use in

line with the WHO recommendation. However, it is still used in a few countries. Interestingly, it is not as reactogenic as Semple vaccine due to the absence of myelin in tissues originating from neonatal animals. However, SMB vaccine is similar to Semple vaccine, especially when it comes to lower potency and the need for administration of multiple doses.

Duck Embryo Vaccine

Virologists made a breakthrough during the 1930s by discovering that an economical and convenient method for cultivating a wide variety of animal viruses was the chick embryo technique; by the 1950s and 1960s chick and duck embryos were being used for vaccine manufacture [37] and they are still used today for the production of vaccines against several agents including RABV [38]. This technique offers several advantages such as availability; ease of handling; presence of naturally sterile environment within the confines of egg components; inability of the embryo to produce antibodies against the viruses used as inocula; and availability of eggs with a relatively uniform genetic constitution. The WHO has been advocating the use of embryonated eggs as a production platform since 1983 [39]. Nevertheless, the use of embryonated eggs poses a number of limitations including the risk of insufficient supply, time-consuming processes with inconsistent yields, high costs of manufacture, and the potential for allergic responses to egg components [40].

The production of modern purified duck embryo vaccine (DEV) involves propagation of the Pitman-Moore (PM) strain of RABV in embryonated duck eggs, extraction of RABV from the brain of infected embryos under mild conditions (without any mechanical forces) to avoid the release of soluble avian antigens that can pose purification hurdles and induce adverse reactions. The purification involves continuous density-gradient centrifugation essentially to remove nonviral lipids and the resulting purified RABV is inactivated using beta propiolactone (BPL). This improved methodology for extraction and purification developed at the Berna Biotech enabled the production of PDEV essentially free from any egg proteins and other materials including myelin basic protein. It is worth mentioning that the classical DEV had myelin basic protein known for its encephalitogenic potential causing allergic encephalomyelitis [41]. The modern DEV is registered and marketed in more than 25 countries. It is immunogenic, safe, and well-tolerated similar to other tissue culture-derived human rabies vaccines [42].

Tissue Culture Vaccines: The Second Generation

Herein, substrate refers to the cultured cells that are used to produce the desired biotechnological/biological products. The ability to perform successive infectious cycles in cell culture was a critical step for research on viral diseases and development of vaccines [43]. Production of vaccines using animal cell substrate was one of the earliest commercial applications of in vitro animal cell technology. Within animal cell substrates, there are a number of cell types that are currently used by manufacturers for the production of human rabies vaccines; primary cells (used without passage in tissue culture); diploid cells (cells with a finite life span and passaged in tissue culture); and continuous cell lines (cells with an infinite life span

and apparently unlimited capacity to replicate). The challenge in standardizing cell substrates is to strike a balance between the desire for a very efficient production system and the goal of minimizing risks. Obviously, manufacturing a safe product necessitates thorough characterization of a cell substrate, validation of the manufacturing process for removal or inactivation of potential adventitious agents and testing of the bulk and final product both for vaccine antigen and impurities. Overall, cell culture-based rabies vaccines have a well-established safety and efficacy profile, irrespective of the type of cell substrate, production system, and purification and formulation strategies, in comparison to previous generation vaccines derived from nerve tissues [44]. Most importantly, cell culture-based rabies vaccines have enabled dosing schedules to be vastly reduced and side effects minimized compared with NTVs [45]. Cell culture is becoming the system of choice for manufacturing many viral vaccines as it offers distinct advantages over egg-based production, which include shorter lead times and greater process flexibility [40].

Cell Substrates for RABV Propagation

Primary Cells

The development of tissue culture systems for virus propagation paved the way for a paradigm shift in vaccine manufacture. Primary cells are derived directly from an animal source. They retain the characteristics of tissues from which they originate and do not have tumorigenic properties. They are characterized by a limited number of subcultures owing to a very short life span of such cultures. Hence, they are not stored, or only stored to a limited extent as cell banks. The most important source of primary cells intended for the production of human rabies vaccines is the avian embryo. For example, chicken embryo fibroblasts (CEFs) are usually derived from a 10-day old embryo after dissection of the head, wings, and body cavity contents [46]. The embryo is enzymatically and mechanically disaggregated. The resulting cell suspension containing CEFs is transferred to appropriate culture vessels for infection. The fertilized eggs intended for use in virus production today are derived from flocks with a well-defined regulatory status, referred to as specific pathogen-free (SPF) sources [43]. However, industrial-scale virus production requires a large number of SPF fertilized eggs [47]. Because of the finite life span of the CEFs, embryonated eggs have to be harvested continuously and each new preparation carries a certain risk of variation in the permissivity of the target virus, inconsistent starting material, and concerns about contamination with potential adventitious agents [48]. These properties introduce undesirable dynamics with complex logistics due to a limited number of vendors who can supply such eggs or can process this substrate into vaccine production [49, 50].

The purified chick embryo cell vaccine (PCECV) is produced using the Flury low egg passage (LEP) RABV strain in CEFs using SPF fertilized eggs, in compliance with currently applicable pharmacopeia and WHO requirements. The Flury LEP RABV is inactivated with BPL, purified via continuous density-gradient centrifugation, resulting in a highly concentrated formulation, stabilized with polygeline and

then lyophilized [51]. The PCECV has comparable immunogenicity and tolerability to that of the human diploid cell vaccine (HDCV), both in animals [52] and human studies [53–56]. It reaches or exceeds the minimum potency requirement of $\geq$2.5 IU per single IM dose. It is known to be very low in human serum albumin (HSA), which is a stabilizer and an important component in most vaccines. The vaccine contains the Flury LEP RABV strain with no deviations from published genetic sequences [57]. The PCECV is marketed globally as Rabipur®, albeit under several brand names in some countries as well [58].

Diploid Cells

Diploid cells are defined as having a finite in vitro life span and contain the full complement of the genetic material, often retain many characteristics of the cell types from which they were derived [48] and are essentially free of tumorigenic properties [59]. The use of human diploid cells for virus propagation, to avoid the difficulties associated with the use of primary tissue culture, is the next major development in cell culture for vaccine production. The essential argument in favor of the use of diploid cell lines for the manufacture of human vaccines was the fact that they undergo senescence and are non-tumorigenic. The human diploid cells have several advantages over primary cells because they allow: multiple expansion passages of material obtained from well-characterized cryogenically preserved master and working cell banks in essentially a closed system [43]; and screening for the absence of adventitious agents. However, they suffer from several disadvantages such as senescence when serially passaged (Hayflick limit); difficulty to upscale in bioreactors especially using microcarriers; and a need for demanding growth medium and difficulty to propagate under serum-free conditions [48].

The two well-known human diploid cell strains, such as WI-38 and MRC-5, serve as the international reference strains [60, 61]. The WHO recommends human diploid cells as one of the safest cell culture substrates for the production of viral vaccines and consequently they have become the preferred cell substrate for vaccine production worldwide. The first tissue culture rabies vaccine originating from primary hamster kidney cells [14, 62] paved the way for the propagation of fixed RABV in human diploid cells. Roughly 3–4 decades ago, most attention focused on the first HDCV, developed at the Wistar Institute in WI-38 human diploid cells [63]. The diploid cells, which originated from a human embryonic lung source, have been extensively tested and used for other viruses as well. The vaccine virus used was the PM 1503 3M strain of fixed RABV derived from a strain originally isolated by Pasteur and maintained by the National Institutes of Health (NIH), USA. In the early 1960s, the virus was adapted to growth in WI-38 cells and was propagated for 52 passages. Subsequently, a master seed pool was prepared in the mid-1960s and the seed was transferred to l'Institut Merieux, a vaccine producing laboratory in 1966. The seed strain was distributed further to Behringwerke in 1969. Early batches of Merieux vaccine were prepared on WI-38 cells. However, later batches composed of whole virion preparations were grown in MRC-5 human diploid cells and inactivated with BPL. The Behringwerke vaccine was concentrated and purified

by rate zonal ultracentrifugation whereas the Merieux product was concentrated by ultrafiltration [64].

Early experimental batches of BPL-inactivated HDCV were immunogenic in laboratory mice and nonhuman primates [65]. When a single dose of concentrated HDCV was administered to a monkey that had previously been inoculated with street RABV, the HDCV gave greater protection than 14 daily doses of DEV or a massive dose of homologous anti-rabies virus serum [66]. Soon afterward, a small-scale human trials were performed on members of the Wistar Institute [67]. Thereafter, clinical trials with HDCV were directed toward establishing optimal regimens for pre- and post-exposure immunization with limited adverse events [68–72]. When 1 ml dose of the Merieux HDCV was used for immunizing volunteers, antibodies were detectable on days 21 and 35 but not day 7 [69]. However, the titers were similar to those of persons given 7 or 12 daily doses of NTV. The highest levels were seen in groups given 4 doses of vaccine on days 0, 1, 2, 3 or 0, 3, 7, and 21. The latter schedule produced the highest levels and a more prolonged response. In 1976, trials in Germany and Iran showed HDCV together with serum or HRIG to afford complete protection to persons bitten by known rabid animals. The vaccination schedule consisted of 6 doses of 1 mL given on days 0, 3, 7, 14, 28, and 90; this regimen was officially recommended by the WHO though in some countries, the day 28 and 90 doses were dropped. Numerous clinical trials and evaluations [68, 73–77] of HDCV have proven its safety and ability to efficiently induce high titers of rabies virus neutralizing antibodies (VNA). These high-quality vaccines, which themselves represent milestones in human vaccine production, permit, with a few injections, prophylaxis for persons at risk. The WI-38 cell strain was used initially, but was switched subsequently to the MRC-5 cell strain, which resulted in the development and licensing of an HDCV in the mid-1970s [78]. The persistence of rabies VNA in persons who received vaccination with HDCV 32 years previously was demonstrated. Further, a single booster inoculation with HDCV resulted in anamnestic responses in all vaccinated subjects [79]. A strong evidence for broad-spectrum cross-neutralization and cross-protection of phylogroup I lyssaviruses using HDCV was shown [44]. From their original inception, the HDCVs have been licensed all over the world. They have been shown to have superior immunogenicity and safety in comparison to purified hamster kidney vaccine (PHKV) [65]. The paramount advantage of primary and finite cells for vaccine production is that an enormous amount of regulatory experience accumulated over several decades since the 1930s.Unfortunately, their worldwide use is limited due to their high cost of production. Continuous cell lines can solve some of the limitations associated with diploid cells, but may introduce other novel challenges [43].

Continuous Cell Lines

The increasing demands in vaccine production yields and safety have urged the development of safer, less expensive, and more efficient cell substrates. Continuous cell lines that originate from animal tissues serve as important cell substrates for the production of various types of biological pharmaceuticals. They have tumorigenic potential and an infinite life span. Nevertheless, a number of studies suggested that

cells below a particular passage number were not tumorigenic [80–83]. In addition, they can be cultured in large-scale fermentors on microcarriers that contribute to standardization, safety, and upscaling of the production system resulting in consistent yields. One of the most frequently utilized mammalian cell lines for vaccine production is the Vero cell line, which was established from the kidney tissue of an African Green Monkey (AGM) [84]. It is a continuous cell line that is widely accepted by regulatory authorities for viral vaccine manufacture [48]. It has pseudo-diploid karyotypes [85] and is non-tumorigenic at low passage number [82, 83]. It is chosen mainly for it achieves high virus yields and batches that lack adventitious agents are available. With respect to cell culture technology, the Vero cell line as a cell substrate is characterized by certain limitations. Its anchorage-dependent nature demands cell culture systems requiring large culture surfaces such as roller bottles, microcarriers, Cell Factories, CellSTACK, CellCubes, and fixed-bed bioreactors.

The purified Vero cell-derived rabies vaccine (PVRV) was introduced into clinical practice several decades ago and was an important step forward in the prevention of rabies [45]. Prior to PVRV, the world depended mainly on either one of the three vaccines viz. HDCV, PCECV, or NTV. When it comes to industrial scalability, PVRV is highly suitable, which is not the case with HDCV. The PVRV (Verorab®) is licensed for use in over 100 countries and over 40 million doses have been administered [86]. Verorab® has been assessed in a large number of clinical situations and studies by considering 0.5 IU/mL antibody titer as the threshold of immunogenicity. This threshold correlates well with protection from clinical rabies [87]. Verorab®, when administered through IM or ID route for PrEP, induces adequate VNA titers, although levels tending to be lower following ID vaccination [86]. However, booster doses lead to a robust immune response irrespective of the route chosen for administration [88, 89]. Verorab® meets the WHO criteria for IM PEP for both the Essen (5 doses; 1-1-1-1-1) and Zagreb (4 doses; 2-1-1) regimens [87] and its immunogenicity remains unaffected by simultaneous administration of RIG [90]. It is better tolerated than HDCV and seems to not induce the allergic-type reactions that can be seen in HDCV recipients [88]. The ease of manufacturing safe, efficacious, and economical rabies vaccines using Vero cell substrates attracted the interest of several manufacturers worldwide including the Human Biologicals Institute, India (Abhayrab®), Bharat Biotech International Limited, India (Indirab®), Liaoning Chengda Biology Co. Limited, China (Speeda®), Serum Institute of India Private Limited, India (Rabivax—S®), etc. A list of various inactivated human rabies vaccines indicated for parenteral route of administration is shown in Table 3.

Next-Generation Cell Culture Vaccines

Vero Cell Line-Derived Vaccine

Composition of cell culture medium has a bearing on potential safety concerns of tissue culture rabies vaccines. Traditional production processes make use of animal-derived substances such as serum, trypsin, and lactalbumin. Serum has some major

Table 3 List of selected inactivated human rabies vaccines indicated for parenteral route of administration

S. No.	Name of the vaccine	Vaccine virus strain	Cell substrate	Viral inactivant	Preservative	Adjuvant	Name of the company
1	Imovax® Rabies	PM/WI38 1503 3M	Human diploid cell	BPL	None	None	Sanofi Pasteur SA, France
2	RabAvert	Flury-LEP	Chicken embryo fibroblast	BPL	None	None	Novartis Vaccines and Diagnostics GmbH, KG, Germany
3	Verorab	PM	VERO cell	BPL	None	None	Sanofi Pasteur SA, France
4	Abhayrab	PV	VERO cell	BPL	Thiomersal	None	Human Biologicals Institute, India
5	Indirab	PM	VERO cell	BPL	Thiomersal	None	Bharat Biotech International Ltd, India
6	Rabipur	Flury LEP	Chicken embryo fibroblast	BPL	None	None	Novartis Vaccines and Diagnostics India
7	SII Rabivax	PM	Human diploid cell	BPL	Thiomersal	Aluminum phosphate	Serum Institute of India Ltd, India
8	Lyssavac-N	PM	Duck embryo	BPL	Thiomersal	None	Zydus Cadila, India
9	COCAV	Wnukowo N32	Primary hamster kidney cell	UV and formaldehyde	None	None	Tchumakov institute, Russia
10	Kaketsuken KNPCEC	HEP-Flury	Chicken embryo fibroblast	BPL	None	None	Astellas Pharma Inc, Japan
11	SPEEDA	PV	VERO cell	BPL	None	None	Liaoning Chengda, China

disadvantages, such as undefined chemical composition; a lot-to-lot variation; transmission of adventitious agents and their by-products such as bacterial endotoxin; variation in terms of growth potentiality; and prohibitive cost. These disadvantages can be circumvented by serum-free approaches for culture of animal cell lines. Such approaches are widely acknowledged because they make downstream processes more straight forward and alleviate safety concerns that shroud the vaccines [91]. In an effort toward continuous improvement of the vaccine production process, at least one producer, Sanofi Pasteur, has developed an improved serum-free, and PVRV-Next Generation (PVRV-NG) vaccine. The PVRV-NG is prepared from the inactivated PM strain of RABV common to Verorab® and Imovax® vaccines. It is produced with the same potency of 2.5 IU but without any components of human or animal origin and antibiotics. In addition to freedom from adventitious agents, it is low in DNA content (100 pg/dose).

The PVRV-NG benefits from the decades of experience gathered from the administration of Verorab® to millions of people in over 100 countries for PrEP and PEP of rabies. Moreover, PVRV-NG is compliant with the European Pharmacopoeia and the specifications defined by the WHO and the United States Food and Drug Administration (USFDA). It was shown to be immunogenic and as safe as Verorab® offering a new alternative for the prophylaxis of rabies [92].

Rabies Virus Strains for Vaccine Production

The RABV strains recommended for vaccine production are "fixed" viruses (as opposed to wild type or "street" viruses) grown in the neural tissue of rabbits, sheep, goats, mice or rats, or in cell cultures, including continuous cell lines. However, the WHO does not recommend or endorse a specific RABV strain for vaccine production [87]. Besides the use of the few proven historical seeds (e.g., Flury LEP, PM, PV, and SAD), derivation of new strains and isolates is encouraged, to maximize safety and minimize costs [93–95].

Authentication of RABV Strains

Lyssavirus genomes are known for their considerable level of variation due to the vulnerability of RNA to accumulate mutation over the years. To date, more than 16 species of the genus *Lyssavirus* have been described [96, 97]. RABV is the commonest causative lyssavirus for human rabies and the only virus used to date in vaccines. Current vaccines may not protect against lyssaviruses other than those in phylogroup I.

The virus strains used for vaccines must be carefully selected, and the antigenic identity of the virus strains used for production should be evaluated periodically. Comprehensive genetic characterization by full genome sequencing of vaccine virus strains is recommended [95]. It is advisable to have genetic characterization as part of the identity of vaccine strains and include this information in the licensing of new vaccines. Ideally, modern techniques such as next-generation sequencing (NGS) should be employed to monitor for subtle genetic changes of vaccine strains over time [98]. As various gene sequences are available in GenBank and various passages of one parental strain may be used, each vaccine manufacturer should sequence their

seed strains and provide individual accession numbers [57]. Care must be exercised not to misinterpret such data as reported elsewhere [99]. It must be borne in mind that nucleotide sequence analysis does not provide information on the efficacy of any RABV strain in use [100]. Interestingly, both sequence comparisons and mismatches with published sequences have been seen among virus strains of commercial human rabies vaccines. Such mismatches could be due to mutational changes after successive passaging; variability among original parent strains; or incorrectness of published sequences. Sometimes, the provider can wrongly declare the parent vaccine strain and the technical dossier can have incorrect records on vaccine strain derivation [57].

Manufacturing Process The need to produce affordable vaccines drives the trend toward large-scale vaccine production [101]. Roller bottles, microcarriers, and multilayer cultivation systems are being used either alone or as a combination for rabies vaccine manufacture worldwide.

Sucrose density-gradient ultracentrifugation (rate zonal ultracentrifugation) and column chromatography attracted the attention of vaccine manufacturers world over for RABV purification. Density-gradient ultracentrifugation is a well-known and established classical purification technique, generally used to purify bulk viral antigens. Briefly, the principle of the method is the separation of particles according to differences in density and thereby, when applied to viruses, to separate them from lighter and heavier cellular material. The main advantage of this technique is the ability to combine the concentration and purification steps in a single unit operation. Also, the technique offers a good resolution for separating full virions from empty capsids, which is very difficult to achieve by chromatography. Although it is theoretically possible to band approximately 10^{15} virus particles in a single run with large-scale continuous ultracentrifugation, from an operational point of view the technique ends up being very laborious and expensive to scale up. On a large scale, density-gradient ultracentrifugation requires high capital and facility investments [102]. Despite these limitations, several manufacturers worldwide prefer rate zonal ultracentrifugation using sucrose for RABV purification. Nevertheless, scalable chromatographic separation technique is a potential alternative for large-scale downstream processing.

Virus purification protocols that are most amenable to scale-up generally involve a chromatographic procedure. Chromatography has been widely used in the downstream processing of virus particles for capture, concentration, and purification of the feedstock using three different arrangements of the stationary phase: packed beds, membrane adsorbers, and monoliths. Packed porous beds of shaped adsorbent particles are widely used in all of the biopharmaceutical industry. Nevertheless, for vaccine purification they suffer from two main disadvantages: limited flow rate imposed by the compromise between pressure drop and mass transfer resistances and, in most cases, low dynamic binding capacity, because the surface available for adsorption under normal contact times is limited to the external surface of the adsorbent particle [103]. Adsorption of viral particles to a solid phase, in fact, is a convenient and practical choice for fractionating and recovering viruses from

impurities originating from cell and culture media. The chromatographic separation is driven by the selective physicochemical interactions between the viruses and closest impurities and the solid phase; the separation can also be based on molecular sieving. While molecular sieving is somewhat imperfect in its selectivity, adsorption methods do offer several important advantages: high flow rates can be used, thus limiting the processing time; biological activity of labile viruses is often preserved since mild conditions are generally used to elute the virus from the chromatographic matrix; scale-up is relatively easy; large volumes of cell lysates can be processed; and the cost of operation is relatively low [104]. However, it is important to remember that the design of suitable selective chromatographic protocols for virus purification must take into account the structure, physical, and chemical surface properties of the viruses [105]. As one example, affinity column chromatography using Cellufine sulfate is widely used since it allows higher recovery of RABV antigens [106].

Formulation

Vaccine formulation development is an important part of the overall developing cycle for producing, testing, and approving new vaccine candidates. Vaccine formulation can be defined as "converting vaccine antigens to medicines" in which the form of commercial dosage not only maintains potency and stability during manufacturing and storage, but also is designed to be administered conveniently to patients from a long-term storage viewpoint, inactivated vaccines are generally more stable and are typically developed as liquid formulations. From a stability perspective, live attenuated vaccines are often freeze-dried (lyophilized) in the presence of a complex mixture of additives and excipients to provide sufficient stability during long-term storage. Freeze-dried vaccines necessitate reconstitution immediately prior to administration and thus require an appropriate diluent [107]. Rabies vaccine as an inactivated vaccine is an exception, in that, commercial vaccines are available in both liquid (adjuvanted) and freeze-dried (unadjuvanted) forms. Freeze-dried and liquid rabies vaccines are ideal for the IM route of administration. However, freeze-dried rabies vaccine is considered suitable for the ID route of administration while liquid rabies vaccine is not [108]. With the ID route becoming more popular because of its cost effectiveness, it is important that freeze-dried vaccines, which can be used by this route, are made widely available and easily accessible [108].

Cross-Protection Against Rabies-Related Viruses (RRVs)

The genus *Lyssavirus* consists of at least 16 different species [96, 97]. On the basis of genetic distances and serological cross-reactivity, the genus has been subdivided into 3 distinct phylogroups: (a) Phylogroup I consists of the species RABV, European bat lyssavirus 1 (EBLV-1), European bat lyssavirus 2 (EBLV-2), Duvenhage virus (DUVV), Australian bat lyssavirus (ABLV), Aravan virus (ARAV), Khujand virus (KHUV), Bokeloh bat lyssavirus (BBLV), and Irkut virus (IRKV); (b) Phylogroup II consists of Lagos bat virus (LBV), Mokola virus (MOKV), and Shimoni bat virus (SHIBV) [95]; and (c) another undefined group consisting of West Caucasian bat virus (WCBV), Ikoma lyssavirus (IKOV), and

Lleida bat lyssavirus (LLEBV) [109]. Of the members of the genus, only 7 species such as RABV, EBLV-1, EBLV-2, ABLV, DUVV, IRKV (phylogroup I), and MOKV (phylogroup II) have caused human deaths [95, 110–115]. The human rabies vaccines manufactured and used worldwide contain fixed RABV strains and are fully protective against known RABV variants (>99%) [116, 117]. However, they could be less effective against infection caused by the remaining lyssaviruses [95, 118, 119]. The HDCV (PM strain of RABV) has been shown to effectively neutralize EBLV-1, EBLV-2, and ABLV all belonging to phylogroup I [44]. Very recently, the PCECV (Flury LEP strain of RABV) has been shown to induce antibodies with the ability to fully neutralize EBLV-1, EBLV-2, ABLV, BBLV, and DUVV, all belonging to phylogroup I and partially cross-neutralize MOKV, a much more distant *lyssavirus* species belonging to phylogroup II [120]. The evidence that supports the ability of rabies vaccine to protect against the other *lyssaviruses* varies widely depending on the vaccine virus strain, challenge method, and host species [44].

The continued discovery of novel *lyssaviruses*, particularly those that are highly divergent from the phylogroup I *lyssaviruses* is a concern for public health, especially amongst those at occupational risk from infection [118, 119]. Regardless of the threat from wildlife, the development of novel vaccines that stimulate a *panlyssavirus* neutralizing immune response is of importance to the population or human subjects at risk [121]. It remains to be seen whether vaccine manufacturers would be willing to develop a multicomponent vaccine of this kind, which would be more complex and potentially more costly than the current modern tissue culture rabies vaccines.

Potency Testing of Rabies Vaccine

The NIH test is currently used to assess the potency of rabies vaccine, a key criterion for vaccine release [122]. This test is based on mouse immunization followed by intracerebral viral challenge. It still remains the reference method for the potency determination of human and animal inactivated rabies vaccines and it is still widely used throughout the world. This test suffers from many disadvantages: it is expensive and time consuming; uses a large number of animals; causes significant animal distress; and suffers from high variability. Recently, the European Pharmacopoeia has recognized the use of a serological potency assay (SPA) as an alternative method to the challenge test [123]. This new test is based on the determination of rabies VNA titers in vaccinated mice, by using a modified Rapid Fluorescent Focus Inhibition Test (mRFFIT). With the objective of adopting this new method for the batch release of inactivated rabies vaccines, its performance was evaluated on a large collection of rabies vaccines. The Fluorescent Antibody Virus Neutralization test (FAVNt) was used in parallel with the mRFFIT, and the results were compared to the mouse challenge test. The results demonstrated that the SPA is capable of estimating the potency of vaccines formulated with a margin well above the minimum of 1 IU/dose. For low potency vaccines, this new method demonstrated some limitations, due to the recurrent invalidation of the assay. FAVNt may be more sensitive when compared to the mRFFIT. In addition, an enzyme-linked immunosorbent assay

(ELISA) as an alternative to the NIH test was developed. This ELISA is based on monoclonal antibodies recognizing specifically the native form of the viral G-protein, the major antigen that induces neutralizing antibody response to RABV. The ELISA was able to distinguish between potent and subpotent vaccine lots. Satisfactory agreement was observed between the ELISA and the NIH test in the determination of the vaccine titers and their capacity to discern conform from nonconform batches. This ELISA meets the criteria for a stability indicating assay and has been successfully used to support the development of a new generation of rabies vaccine candidates. After an European Partnership for Alternative Approaches to Animal testing (EPAA) international pre-collaborative study, this ELISA was selected as the assay of choice for the European Directorate for the Quality of Medicines (EDQM) collaborative study aimed at replacing the rabies vaccine NIH in vivo potency test [122].

Vaccination

PrEP

Rabies is one of the most feared zoonotic diseases because it has the highest human—case fatality proportion of all conventional infectious diseases [45]. Fortunately, they happen to be fatalities can be prevented by vaccines. Viral clearance before the manifestation of illness is critical; this, in turn, relies on the presence of VNA. Therefore, the prevention of rabies depends mainly upon rabies vaccines capable of inducing VNA quickly [124]. The method of protection is likely to be a combination of local virus neutralization by antibodies or via antibody-mediated clearance of virus-infected cells [125]. The PrEP is usually given through the IM route on days 0, 7, and 21 or 28. According to the WHO, ID vaccination is seen as an economical and acceptable alternative to the IM route, but it is technically more demanding, requires appropriate staff training and qualified medical training.

PEP

Globally, rabies occurs on all continents, in more than 150 countries and territories [126]. More than 3 billion people live in areas in which rabies is enzootic [28]. Worldwide, millions of exposures are registered, resulting in tens of thousands of human deaths, with most occurring in Asia and Africa. Based on the types of interaction with suspected rabid animals, exposure is broadly classified into three categories I, II, and III. All exposures determined to represent a risk for rabies require PEP, which includes immediate local treatment of all bite wounds and scratches with thorough washing and disinfection, local wound infiltration with RIG (for category III alone) and vaccination. The main purpose of PEP is to prevent the development of clinical rabies after exposure has occurred. The combination of active and passive immunization is considered the status quo for PEP, except for those persons who have been previously immunized with a rabies vaccine via a recognized schedule and a WHO approved vaccine [95]. Annually, more than 15 million people worldwide are estimated to receive PEP, which prevents hundreds of thousands of rabies deaths [27]. While the combination of rabies vaccine and RIG is nearly 100%

effective in prevention before illness, attempts to use rabies vaccine or RIG after the onset of symptomatic rabies have not been proven beneficial [127]. On the basis of a model experiment using hamsters, vaccine injection at the wound site in the same manner as administration of RIG provided protective efficacy that was not inferior to the current optimal PEP, a combination of vaccination and RIG. However, only further studies can determine whether this protocol and other modalities (such as cytokine administration) can replace the use of RIG [128].

Vaccination for Special Scenarios

In reality, all individuals are equally vulnerable for exposure to RABV irrespective of the age, nutritional status, immune competence, physiological status, etc., when they visit or live in rabies endemic regions. However, not everyone can be conferred with active immunity, because vaccination necessitates immunocompetent subjects with a mature immune system. Nevertheless, this is not the case when it comes to conferring passive immunity, which involves transfer of preformed antibodies that can mediate the desired pharmacological action independent of the recipient's immune system [129]. There are scenarios wherein the immune system of human subjects is either suppressed or compromised (e.g., HIV/AIDS patients) with characteristically low $CD4^+$ T cell counts, who usually mount a low or no detectable VNA response to RABV. In these patients and others in whom the presence of immunological memory is no longer assured, proper, thorough wound treatment and antisepsis accompanied by local infiltration of RIG (HRIG/ERIG), and a complete series of 5 IM doses of rabies vaccine (cell culture/avian embryo based) is recommended for category II and III exposures [95]. This is in striking contrast to immunocompetent subjects who receive such a combination of RIG and rabies vaccine only in the case of category III exposure. According to the WHO, in such cases it is advisable to ascertain the requirement for additional dose of rabies vaccine by measuring the VNA response 2–4 weeks after vaccination, where feasible.

There are other special scenarios such as pregnancy (vaccination is risky because of the perception of teratogenesis); malnutrition; young age (vaccination is ineffective) with immature/impaired immune system; and organ failure (vaccination is ineffective) with associated immunosuppression [130]. According to the WHO, PEP is not contraindicated in the above-mentioned special scenarios due to the fatal nature of rabies and lifesaving nature of vaccine. There are reports to demonstrate both success [131] and failure [132] of PEP in HIV/AIDS patients. Similarly, PEP is safe and effective during pregnancy [133] and in malnourished children [134, 135]. In one report, a kidney failure patient undergoing hemodialysis failed to develop a primary response after PEP, which could, however, be mitigated when higher amounts of antigen were used [136]. Nevertheless, research on the safety and efficacy of PrEP in the above-mentioned special scenarios seems to be a road less traveled. Any effort taken in this regard deserves full financial support from the funding agencies and cooperation from the medical fraternity and health officials.

Adverse Events

It is not uncommon to encounter adverse events (AE) after vaccination. Probably, there is no vaccine known to date which is completely free from causing adverse reactions. However, vaccines differ from each other in terms of the number and degree of adverse reactions. Interestingly, several adverse reactions are common to nearly all vaccines. In contrast, there are some adverse reactions that are vaccine specific. Vaccine ingredients are basically responsible for many adverse reactions. Components that may elicit an allergic response include active immunizing antigens, conjugating agents, preservatives, stabilizers, antimicrobial agents, adjuvants, and culture media used in the preparation of vaccines [137]. The proteins most often implicated in vaccine allergies include egg, gelatin, and yeast [137, 138].

In general, all vaccines contain both active and inactive ingredients, both of which can cause adverse reactions, including anaphylaxis and allergy. These ingredients include foreign proteins (Bovine Serum Albumin [BSA], Human Serum Albumin [HSA]) used during manufacture, viral inactivant (BPL), preservative (Thiomersal), stabilizer (HSA), adjuvant (aluminum phosphate gel), antibiotics (neomycin), and antigen itself [139]. Four types of hypersensitivity reactions, such as types I, II, III, and IV are known, of which types I to III play a role in connection with vaccination [140]. Addition of HSA during RABV antigen production and to the finished product essentially as a stabilizer is a common practice [141, 142]. This practice should be in line with international requirements for blood, blood components, and plasma derivatives as well as guidelines on human TSEs as strongly recommended by the World Health Organization (WHO) and European Medicines Agency (EMA) [57]. Extreme care must be taken such that the HSA in the final product does not undergo much change and therefore will not be able to cause adverse reactions in the recipients [143, 144]. In addition, there are concerns about HSA with regard to its possibility of transmitting adventitious agents [139, 145, 146]. Routine use of HDCV caused hypersensitivity reactions belonging to type I [146–148] and III categories [149] attributed mainly to the presence of BPL-modified HSA with the capacity to induce IgE (type I) and a tendency to form immune complexes (type III). In addition, HSA can suppress the induction of Tumor Necrosis Factor (TNF) production by vaccine [150]. The risk of type I and III hypersensitivity reactions can be mitigated by inactivating RABV using BPL following removal of HSA using appropriate purification techniques [151–153].

The PCECV originating from primary CEFs contains micrograms quantity of egg proteins and is therefore contraindicated in patients who are hypersensitive to eggs [154]. If a rabies vaccine is needed for an egg-hypersensitive patient, alternative vaccines, such as the HDCV and PVRV that contain no egg components, are preferred. Alternatively, such patients may be tested for egg hypersensitivity by means of a prick test to observe if there is a cutaneous response. If the test has negative results, the PCECV may be administered in a graded manner in a clinical setting in which anaphylaxis can be recognized readily and managed. Gelatin has been implicated in both IgE- [137] and non-IgE-mediated allergic reactions to vaccines [155]. The PCECV has less than 12 mg of gelatin and beef and pork meat sensitized children having IgE antibodies tend to cross-react with gelatin

[155, 156]. However, appropriate use of desensitization procedures will minimize the risk of anaphylaxis and many gelatin allergic patients are able to receive vaccines containing gelatin when medically necessary.

Awareness of the potential for IgE-mediated sensitivity to rabies vaccines and their components is important. This allows the appropriate use of desensitization protocols with the safest vaccine product available, to minimize the risk of anaphylaxis and permit the full benefit of the vaccine to prevent disease [157]. Patients with a history of gelatin hypersensitivity should be evaluated by an allergist or immunologist before administration of a gelatin-containing vaccine [158]. As mentioned above for PCECV, an obvious alternative strategy to mitigate this problem is to choose a rabies vaccine known to be devoid of gelatin. Age-dependent incidence and severity of AEs vary depending on the rabies vaccine and it is imperative to assess accurately the safety of various rabies vaccines for people at different ages. The safety of 4 types of rabies vaccines for people at different ages who received PEP after WHO category II animal exposure was compared [159]. The only difference between the vaccines was the manufacturing technique, i.e., fermenter and spinner bottle. In early periods, vaccines were made by cell cultivation in roller bottles. With the improvement of biological products and technology, application of bioreactor systems emerged and gradually replaced the traditional process [160]. The numerous advantages of a bioreactor mode include simple operation, high volumetric productivity, and low costs [161]. Most importantly, the vaccines produced in bioreactors are much safer than those in roller bottles because of great reduction of residual cell protein, cell DNA, and bovine serum [162, 163]. In other words, advanced manufacturing technology was responsible for the relatively lower incidence of AEs. Thus, manufacturing technique was another important factor that had influence on vaccine safety, especially for children younger than age 5 years. The vaccines that were produced by biological fermentation and administrated with a small dose have comparative higher safety in children at risk of RABV exposure [159]. In summary, rabies is a life-threatening disease, and the benefits of vaccination far outweigh the risks in persons exposed or potentially exposed to the virus. Most AEs are nonserious and have been described previously [164]. PCECV is as safe as PVRV and can be used as an alternative when PVRV is not accessible [165].

Oral Vaccines

Wildlife are maintenance hosts of RABV. Therefore, it is essential to break the chain of transmission to control rabies in wildlife. Poisoning or trapping to control the movement of the disease in the species of concern was attempted early on. However, the vaccination of wildlife against rabies became attractive after the poisoning and trapping strategies failed repeatedly. Oral rabies vaccination (ORV) is a socially acceptable disease control method for wildlife reservoirs. The initial requirements of such a vaccine were safety, efficacy, and low cost, allowing animals to get immunized upon oral uptake of rabies vaccine-laden baits [166]. The distribution of vaccine-laden baits for wildlife is intended to interrupt the transmission from rabid

animals to healthy ones, and ultimately to eliminate RABV from those vectors. Vaccination coverage of approximately 70% of the vector population is estimated to be sufficient to block viral transmission [167].

Wildlife

The WHO recommends that rabies vaccines should not cause any AE in target and nontarget species [88]. The safety of rabies vaccine candidates should be evaluated in rodents, wildlife, and domestic species [168]. ORV using modified-live RABV has been highly successful in different reservoir species. The first animal targeted was the European red fox (*Vulpes vulpes*) followed by the raccoon dog (*Nyctereutes procyonoides*) [169]. Subsequently, the concept of oral rabies baiting was investigated for other animal species, including raccoons (*Procyon lotor*) [170, 171], coyotes (*Canis latrans*) [172, 173], gray foxes (*Urocyon cineroargenteus*), striped skunks (*Mephitis mephitis*) [174], small Indian mongooses (*Herpestes auropunctatus*) [175, 176], and domestic dogs (*Canis lupus domesticus*) [177–179]. ORV has eliminated rabies in 12 European countries [180–182] and is currently being used in the majority of rabies affected European countries [183]. Not all animal species respond equally well to vaccination by the oral route; some species like the striped skunk seem to be extremely refractory to ORV, irrespective of the construct or the amount of virus present in the bait. The seroconversion rates in raccoons upon ORV are lower than in gray foxes and coyotes. These could be due to two reasons; vaccine is not as immunogenic in raccoons suggesting the need for an adjuvant; and/or vaccine spillage suggesting the need for a more viscous vaccine mixture. N,N,N-trimethylated chitosan (TMC) increases the viscosity of the vaccine and potentially acts as an adjuvant to improve the immune response in raccoons (*Procyon lotor*) [184]. Wolves may not easily take commercial baits; goat meat baits seem to have the highest uptake compared to rodent and intestine baits [185]. Recombinant vaccines using replication-competent adenoviruses as vectors, e.g., ONRAB is one of the recombinant oral rabies vaccines that use a human adenovirus vector to express the RABV G protein [186, 187]. The adenovirus system allows for the generation of high titer recombinant vector for delivering the gene encoding the rabies virus glycoprotein to cells which then upon transcription and translation induces a VNA response. IM immunization with the Ad-0910G and Ad-0910N viruses in raccoon dogs was safe and induced high neutralizing antibody titers. These results, together with safety and immunogenicity in raccoon dogs, make the combined Ad-0910G and Ad-0910N strains an alternative to the attenuated rabies vaccine used previously for animal rabies control. Preparation and application of the concentrated Ad-0910G strain, which exhibited a viral titer over $10^{10}TCID_{50}$ (Median Tissue Culture Infective Dose)/mL, to raccoon dogs results in high VNA titers. Additionally, further study concerning the effectiveness of ORV in accordance with the National Standard Assay for Veterinary Biologic Products in dogs and raccoon dogs is needed [188]. A recent study threw light on the existence of diversity of variants in oral rabies vaccines widely used in Europe as well as the presence of a mix of at least two different variants in all tested batches. Such an investigation may also reveal the potential reversion to a virulent form and the possible identification of

shifts in virus populations during the vaccine manufacturing process. The results demonstrate the need for vaccine producers to use new robust methodologies in the context of their routine vaccine quality controls prior to market release. Since RNA viruses are naturally composed of diverse quasispecies populations, a stability study of attenuated live rabies vaccines, coupled with a virus population study using NGS technology, could help to better understand live vaccine attenuation processes [189]. Deep sequencing analysis of viral vaccines was proposed to test the identity and stability of modified-live viral vaccines, e.g., for quality assurance during batch release procedures. It is to be preferred over consensus sequence analysis and allows for routine integration of deep sequencing data in vaccine quality control and licensing for highly reliable assessment of strain identity and stability [98].

Domestic Animals

The purpose of rabies vaccination in domestic animals is mainly to protect individual animals when they are exposed to RABV not only from the urban cycle but also the sylvatic cycle. To eliminate rabies from dogs in an endemic area, at least 70% of the population needs to be vaccinated during annual rabies mass vaccination campaigns [190]. Parenteral vaccination is the method of choice for owned dogs (i.e., dogs with a person that claims responsibility, according to the World Organisation for Animal Health (OIE) definition) and is therefore more cost-effective measure in preventing human rabies [191]. Catching free-roaming dogs is easier if they are owned. Therefore, dog ownership is an important factor in determining the percentage of dogs vaccinated during a campaign [192]. The parenteral vaccination of stray or owned but uncontrolled dogs is more difficult, laborious, and expensive. In contrast, ORV has the potential to mitigate issues associated with parenteral vaccination. In addition, international guidelines for rabies control in dogs and implementation of field trials using oral vaccines are available. The cost of ORV is higher than that of the parenteral vaccination. This could be reduced by the use of aerial distribution in certain well defined and restricted areas, but "hand-out" models to dogs may be the most practical form of delivery due to continued concerns about safety in nontarget species [193]. A combination of parenteral and oral vaccination may help to increase the vaccination coverage in the canine population which could lead to rabies elimination.

Currently, two oral rabies vaccines SAG-2 and VR-G are recommended by the WHO for dog vaccination. VR-G, a recombinant Vaccinia virus expressing RABV glycoprotein (G) has been successfully used for control of fox rabies in Europe and coyotes and raccoons rabies control in the USA. SAG-2 is an attenuated RABV derived from the SAD-Bern strain (B19) with 2 nucleotide mutations at its glycoprotein codon 333. SAG2 has been widely used in Europe and led to wildlife rabies elimination in several European countries. It should be noted that no vaccine-induced rabies cases were reported in Europe after the distribution in the environment of more than 20 million doses of SAG2 baits. This vaccine is registered for the control of canine rabies in India and has been mainly evaluated in Tunisia, Mexico, South Africa, and Indonesia, demonstrating its efficacy for dog vaccination in the field. The level of VNA induced by SAG-2 is generally low in dogs after ORV and

Table 4 List of selected animal rabies vaccines indicated for the oral route of administration

S. no.	Product name	Produced by	For use in	Route of vaccination	Cell culture
1	FUCHSORAL	IDT Biologika GmbH, Germany	Red fox	Oral	Cell culture
2	LYSVULPEN	BIOVETA, Czech Republic	Red fox	Oral	Cell culture
			Raccoon dogs	Oral	Cell culture
3	RABIGEN ORAL	VIRBAC S.A, France	Red fox	Oral	Cell culture
			Raccoon dogs	Oral	Cell culture
4	RABIDOG ORAL	VIRBAC S.A, France	Dogs	Oral	Cell culture

not all dogs develop detectable VNA. Recombinant attenuated RABV expressing dog Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) could induce significantly higher VNA titers after ORV in dogs [194]. The safety and immunogenicity of a newly constructed rabies vaccine strain (ERAGS strain) were assessed by administering via oral and IM routes to growing pigs and sows. No pig inoculated with ERAGS exhibited any clinical sign of rabies over 28 days, and RABV was not detected in tissue samples by FAT or RT-RCR, suggesting that the ERAGS strain may be safe in pigs. Additionally, the vaccinated pigs developed significant VNA titers against RABV, indicating that the ERAGS strain may be immunogenic in swine. Thus, the ERAGS strain is a new, prospective candidate for a rabies vaccine for pigs [195]. In the future, reverse genetics applications may offer even safer and efficacious RABV vaccines for a broader range of species [196]. A list of several animal rabies vaccines indicated for the oral route of administration is found in Table 4.

Monitoring Oral Rabies Vaccination

The effectiveness of ORV is regularly assessed through direct observation, in live-trapped and hunted animals from vaccinated areas [197], through assessment of biomarker (e.g., tetracycline, iophenoxic acid) levels incorporated in the vaccine bait to verify bait uptake and serological status by quantifying RABV-specific VNAs to demonstrate adequate responses to immunization [183]. The two current OIE-prescribed serological reference methods, the FAVNt and the RFFIT [198], are based on cell culture and are therefore sensitive to any cytotoxic products and contaminating agents present in field samples [199]. By comparison, ELISAs are less time-consuming, easier techniques and preferred for assessing the serological response in countries carrying out mass vaccination of dogs and oral vaccination of wildlife [200, 201]. Such ELISA and neutralization methods measure different antibody functions. Rabies antibodies are generally screened from the field via animal cadavers, whose body fluids are often of poor quality. Therefore, the use of alternative methods, such as the ELISA, has been proposed to improve the reliability of serological results obtained on wildlife samples. ELISA has been shown to be a

reliable tool for detection of rabies-specific VNA in the context of evaluation of ORV of foxes from poor quality samples as a substitution for virus neutralization tests and also in dog and cat sera [202]. As a substitution for serum, fluid from the thoracic cavity or extracts from muscle can be used as samples [203].

Future Prospects

- The ID route of immunization and special devices to accomplish administration may become more popular among clinicians to achieve better patient compliance.
- Classical rabies vaccine manufacture is likely to be dominated by bioreactor based mass cultivation of cells, essentially free of raw materials of animal and human origin. These products will be governed by revised regulatory guidelines in terms of advanced molecular techniques for vaccine strain authentication for licensure and ELISA-based potency testing for batch releases.
- Wildlife rabies control programmes may witness changes in terms of vaccine virus strain authentication, bait design for better uptake by target hosts, and improved monitoring of ORV through measurement of serological responses using appropriate methods and enhanced sample collection.
- PEP involving direct inoculation of rabies vaccine into the rabid animal bite wounds may be practised.
- Rabies vaccines with novel and versatile adjuvants may attract wider interest and hit the market place sooner than later.
- Novel VLP-based rabies vaccine without adjuvants may attract the attention of vaccine manufacturers and investors as inexpensive, safe, and effective means of prevention in endemic regions of the globe.
- Human vaccination will remain fairly conservative compared to domestic animal vaccination, which will continue to span the gamut from modified-live to inactivated products and recombinant vaccines.
- Regardless of the enactment of any of these predictions, all of the tools are currently available to prevent human deaths, eliminate canine rabies, and control disease in mesocarnivore populations by oral vaccination.

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Rabies Vaccines for Wildlife

T. Müller and C. M. Freuling

Abstract

Since the first proof of principle experimental study in the 1970s, oral rabies vaccines have gained a great reputation in controlling and eliminating rabies in wildlife. Starting with classically attenuated virus vaccines derived from only a few virulent rabies virus field isolates, oral rabies vaccination (ORV) for the first time has offered new opportunities and opened new avenues worldwide in fighting this fatal zoonosis in particular in its canine wildlife reservoir hosts. Beyond classical approaches, biotechnological tools have been increasingly used to generate new oral rabies vaccines with the aim to improve safety, immunogenicity, and efficacy in the various canine wildlife reservoir species. While heterologous vector systems have been developed for expression of the immunedominant glycoprotein (G) of rabies virus (RABV), recent advancements in recombinant DNA technology and virus reverse genetics helped facilitating vaccine development through targeted modifications and directed attenuation of rabies virus constructs. Unlike in humans, pets, and livestock, there is no alternative way yet to efficiently vaccinate canine wildlife reservoir hosts at a population level other than using modified live virus vaccines. In contrast to any other vaccines for veterinary use, next to safe and efficacious vaccine constructs, attractive species-specific baits, as well as a well-defined distribution system/strategy, are indispensable components of any oral rabies vaccine for wildlife. As baits play a decisive role for successful application in the field they are an integral part of the licensing procedure. This chapter summarizes the state-of-the-art information on oral rabies virus vaccines for wildlife and provides an outlook on the challenges of vaccine development for wildlife for the future.

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H. C. J. Ertl (ed.), *Rabies and Rabies Vaccines*,
https://doi.org/10.1007/978-3-030-21084-7_3

Introduction

Vaccines remain one of the greatest accomplishments of human ingenuity, scientific endeavor, and combined global efforts of public and animal health communities [1]. Vaccines are biological preparations primarily developed to protect from disease or infection by pathogenic agents. They typically contain weakened, killed, or modified forms of the respective agent, surface proteins or its toxins, which stimulate the natural defense mechanisms of humans and animals alike to develop active acquired cellular and/or humoral immune responses to prevent infection with field strains.

There has been growing international recognition of the importance of safe and efficacious vaccines and other interventions as part of the package to combat viral and bacterial diseases in humans. Vaccines should be designed to prevent infection rather than to prevent clinical signs of disease and should, wherever possible, produce sterile immunity [2]. Next to Edward Jenner's innovative contribution to immunization representing the first successful attempt to control an infectious disease by the deliberate use of vaccination [3, 4], the field of vaccination and vaccine development, in general, has also been decisively influenced by early rabies research. Since millennia rabies, the oldest known but neglected viral zoonosis to mankind, poses a serious public health threat throughout the world. Louis Pasteur's first human vaccination in 1885 with a crude rabies virus attenuated by exposure to dry air [5] is considered the foundation for today's efficient pre- and postexposure rabies prophylaxis in humans and preventive vaccination in animals.

As for the latter, the control of viral and bacterial diseases in animals using vaccination has become a global focus of attention to prevent the economic, animal welfare, and public health costs due to severe disease outbreaks in livestock, pets, and wildlife [6]. Vaccines for veterinary use cannot only be applied to protect animal health but also human health from zoonotic infections as exemplified by vaccination of animals against rabies [2, 7, 8]. Considering the fact that vaccination has not only positive immunological impacts at the individual level but also at a herd level, mass vaccination of dogs, the predominant vector for human rabies exposures, with potent inactivated vaccines resulted in elimination of dog-mediated rabies in Europe, North America, Japan, and many other islands [9–11]. Also, Latin America serves as a blueprint for successful reduction of human dog-mediated rabies by mass vaccination campaigns [12], paving the road to the internationally agreed goal of eliminating human dog-mediated rabies cases by 2030 [13].

However, besides the dog as the only recognized domestic reservoir host for rabies virus (RABV), multiple lineages of this virus circulate and have coevolved in a wide range of wild mammalian hosts primarily within the Carnivora order involving Canidae, Procyonidae, Herpestidae, Mephitidae, Viverridae, and Mustelidae [14, 15] (Table 1). However, Chiroptera is also the primary host of RABV in the Americas [44]. As in many areas of the world, wildlife rabies surveillance is nonexistent, the list of wildlife reservoir species will most likely rapidly expand with increasing surveillance intensity in the near future. A good example is the

Table 1 Principal animal reservoir hosts of rabies within the order Carnivora throughout the world

Geographic region	Species	Scientific name	Family	References
Arctic	Arctic fox	*Vulpes lagopus*	Canidae	[16–18]
Europe	Red fox	*Vulpes vulpes*	Canidae	[19–21]
	Racoon dog	*Nyctereutes procyonoides*	Canidae	[21]
Africa	Striped jackal	*Canis adustus*	Canidae	[22]
	Black-backed jackal	*Canis mesomelas*		[23]
	Bat-eared fox	*Otocyon megalotis*		[24]
	Cape gray mongoose	*Galerella pulverulenta*	Herpestidae	[25]
	Yellow mongoose	*Cynictis penicillata*		
	Banded mongoose	*Mungos mungo*		
North America	Raccoon	*Procyon lotor*	Procyonidae	[26, 27]
	Striped Skunk	*Mephitis mephitis*	Mephitidae	[26, 27]
	Red fox	*Vulpes vulpes*	Canidae	[28]
	Coyote	*Canis latrans*		[29, 30]
	Grey fox	*Urocyon cinereoargenteus*		[30]
Caribbean Islands	Small Indian mongoose	*Herpestes auropunctatis*	Herpestidae	[31]
South America	Crab-eating fox	*Cerdocyon* sp.	Canidae	[32]
Middle East	Red fox	*Vulpes vulpes*	Canidae	[20, 33, 34]
	Golden jackal	*Canis aureus*		[35]
Asia—Far East	Red fox	*Vulpes vulpes*	Canidae	[36, 37]
	Steppe fox	*Vulpes corsac*		[38, 39]
	Raccoon dog	*Nyctereutes procyonoides*		[37]
	Ruddy mongoose	*Herpestes smithii*	Herpestidae	[40]
	Chinese ferret badger	*Melogale moschata*	Mustelidae	[41, 42]
	Golden palm civet	*Paradoxurus zeylonensis*	Viverridae	[43]

marmoset (*Callithrix jacchus*), which was identified as a new wild rabies reservoir in Brazil [32].

Until the 1970s, the control of wildlife rabies in Europe and North America by vaccination seemed inconceivable. In the hope of disrupting the natural chain of infection, conventional methods of disease control exclusively aimed at a drastic decimation of the population density of wildlife reservoir hosts [45]. Apart from a few exceptions, decade-long painful experience showed that all those measures including intensive culling, poisoning, and hormonal sterilization were unable to bring the disease under control on a larger scale, or, in contrast, were counterproductive [10, 45]. As a logical consequence and inspired by the success in dogs since

the early 1970s, the emphasis for control of wildlife rabies shifted toward vaccination of the respective principal reservoir hosts [46]. However, many obstacles had to be overcome. Generally, while the effect on the individual animal determines a vaccine's efficacy, the effect at the population level determines its effectiveness [6]. Although inactivated vaccines induce an adequate immune response in wild carnivore rabies reservoir hosts [47, 48], they are no true alternative as their parenteral application per se precludes their use for mass vaccination of wildlife and therefore, have only been used in trap–vaccinate–release (TVR) campaigns [49, 50].

Since the first proof-of-principle experimental studies in the 1970s [51–54], oral rabies vaccines have opened new avenues for fighting rabies in wildlife. Spanning from classical attenuated to biotechnology-derived approaches, a variety of oral rabies virus constructs for use in wildlife have been developed. All successful approaches are based on replication-competent viruses, as the oral application of inactivated rabies vaccines was ineffective [46, 55].

Attenuated Rabies Virus Vaccine

Several live-attenuated rabies virus vaccine strains have reached market authorization for oral use in wildlife during the past four decades. Figure 1 summarizes the presumed ancestry and passaging history of available live-attenuated rabies vaccine strains. Almost all these vaccine strains are "descended" from only one parent strain designated as "Street Alabama Dufferin" (SAD) isolated from a rabid dog in Alabama, USA, in 1935, and initially exclusively propagated in mouse brain [56]. However, the close genetic relationship of the vaccine strains Pasteur virus and SAD B19 as a representative of SAD-related vaccines is somewhat striking [57], and may, in fact, question the origin and passage history of the respective vaccine strains. At this time, the former Centers for Disease Control (CDC) of the United States was located in Montgomery, Alabama. So, the word "Alabama" may just as well refer to the laboratory rather than to the origin of the dog.

Subsequent serial passaging of SAD in various non-neural cells, e.g., hamster kidney cells, embryonated chicken eggs, and pig kidney cells, resulted in the highly attenuated, cell culture-adapted "ERA" strains [56, 58, 59]. The derived high-titered commercial ERA vaccine was shown to be efficacious in red foxes after immunization by the oral route [51–54]. The ERA vaccine strain was further developed by enhanced adaptation to baby hamster kidney (BHK) cells and additional thermal stabilization yielding a vaccine virus strain that was subsequently renamed "SAD Bern" (SAD $\text{Bern}_{\text{orig}}$) when transferred to Europe [60].

Both the ERA and the SAD $\text{Bern}_{\text{orig}}$ strains (Fig. 1) are the progenitors of the great majority of today's advanced forms of ERA- and SAD-based live-attenuated oral rabies vaccines for wildlife of the 1st generation. Some of these 1st-generation vaccines contain descendants generated from continued passaging and adaptation on original BHK cells or cloned BHK/BSR cells, e.g., ERA-BHK21 vaccine [61, 62], Vnukovo-32 [63], Bio-10-SAD Bern [64], SAD B19 [65], SAD P5/88 [66], and

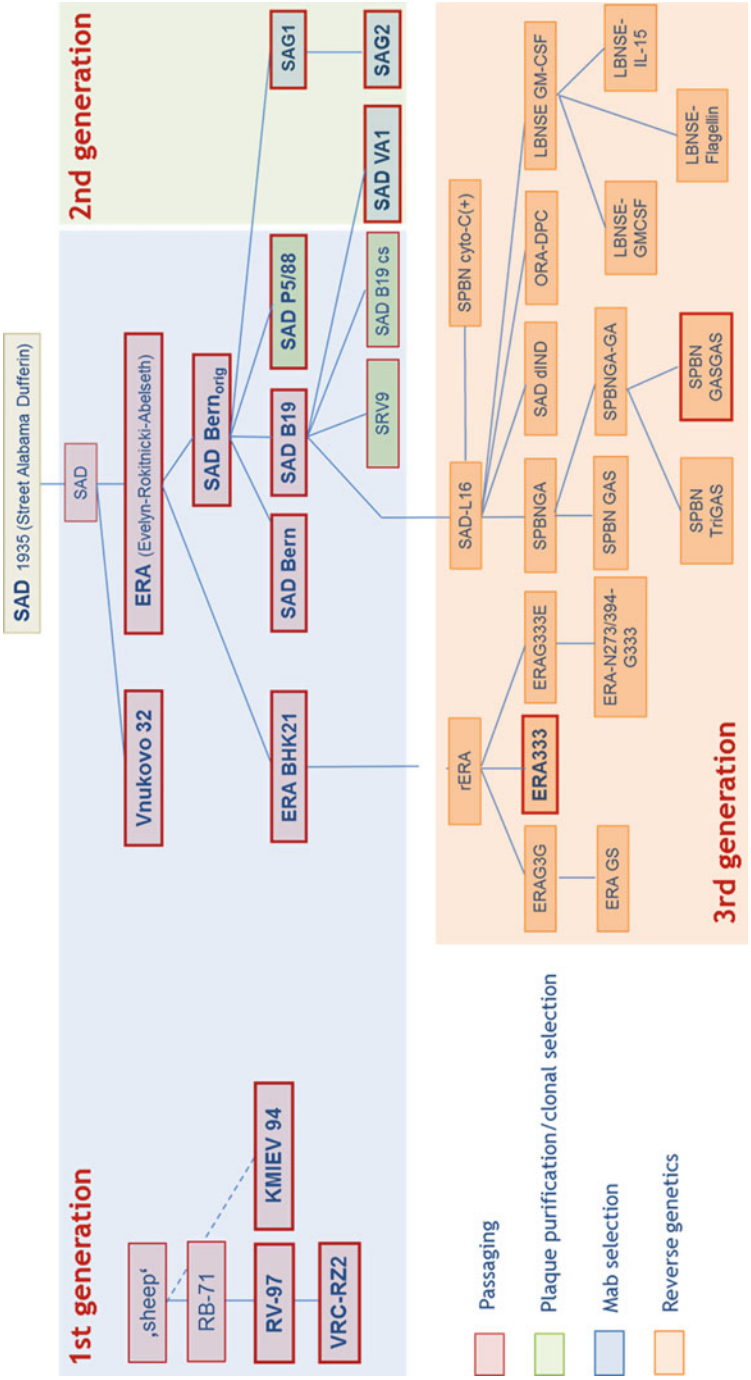

Fig. 1 History and evolution of 1st-, 2nd-, and 3rd-generation attenuated rabies virus vaccine strains. Vaccine strains highlighted (framed) have been licensed as vaccines and/or were used in the field. The methods used to generate the respective viruses are indicated (see legend)

SRV9 [67]. Other 1st-generation vaccines for oral vaccination of wildlife but of different ancestry are the Russian vaccine strain RV-97 and strain VRC-RZ2 from Kazakhstan. Interestingly, RV-97 and its attenuated precursor viruses RB-71 and "Sheep" are phylogenetically more related to the Japanese group of vaccine strains such as the Nishigara strain [68]. High sequence identity (99%) of the gene encoding for the nucleoprotein (N) of RABV suggests strain VRC-RZ2 from Kazakhstan to be a direct descendant from RV-97 [69]. According to passage history, the Belarussian vaccine strain KMIEV-94 is a derivative of rabies virus strain 71-Bel-NIIEV VGNKI obtained through serial passages in different cell cultures [70]. Whether both strains VRC-RZ2 and KMIEV-94 can be traced back to the same ancestor strain (RB-71) remains to be proven. This group of vaccine strains has been used for oral rabies vaccines for wildlife in countries, such as Kazakhstan, Belarus, and Ukraine [68, 71].

In response to safety concerns of 1st-generation vaccines [15, 72] associated with experimentally known residual pathogenicity in naïve and immune-compromised rodents [65, 73–75], the safety profile was improved by generating selection mutants using anti-G monoclonal antibodies. As a result, vaccine strains SAD VA1, SAG 1, and SAG 2 were obtained [76–79]. The latter is characterized by a replacement of arginine with glutamic acid at residue 333 of the RABV G (G333E) that further reduces pathogenicity [77]. However, 2nd-generation oral rabies vaccines are also not completely apathogenic as was shown in immunocompromised and inbred mice [80, 81]. While the close genetic relationship of SAD-derived 1st- and 2nd-generation oral rabies vaccines has been confirmed both by full genome sequencing and next-generation sequencing [82, 83], the latter revealed that SAD-derived oral rabies vaccines may consist of more or less heterogenous viral populations [83, 84].

Between 1978 and 2017, more than 1 billion baits containing different oral rabies vaccines were distributed in Europe and North America, with the number of 1st-and 2nd-generation oral rabies vaccine baits amounting to about 720 million (Fig. 2). In contrast to North America, where only a single attenuated vaccine strain (ERA-BHK21) had been deployed in ORV campaigns in the period between 1989 and 2004 that eliminated rabies from eastern Ontario, Canada [62], ten different 1st- and 2nd-generation oral rabies vaccines were applied for ORV throughout Europe during this time period with SAD B19 and SAD Bern having been the most widely used vaccine virus strains so far (Fig. 2, [85]).

Throughout the entire period, 23 vaccine-induced rabies cases have been reported from oral vaccination areas in red foxes and also nontarget species from both Europe and North America for ERA-BHK21 [62], SAD Bern [86–89], SAD B19, and SAD P5/88 [86, 89–91] resulting in an incidence rate of 1 in 48 million vaccine doses distributed [85]. Recent in-depth analyses using next-generation sequencing revealed the viral population in those vaccine-induced cases to be clonal in contrast to their parental vaccines indicating the presence of a strong bottleneck during infection rather than a reversion to virulence [86]. Vaccine-induced rabies cases have to be reported in the frame of pharmacovigilance. However, none of these cases were connected in time and space or became established within the target species

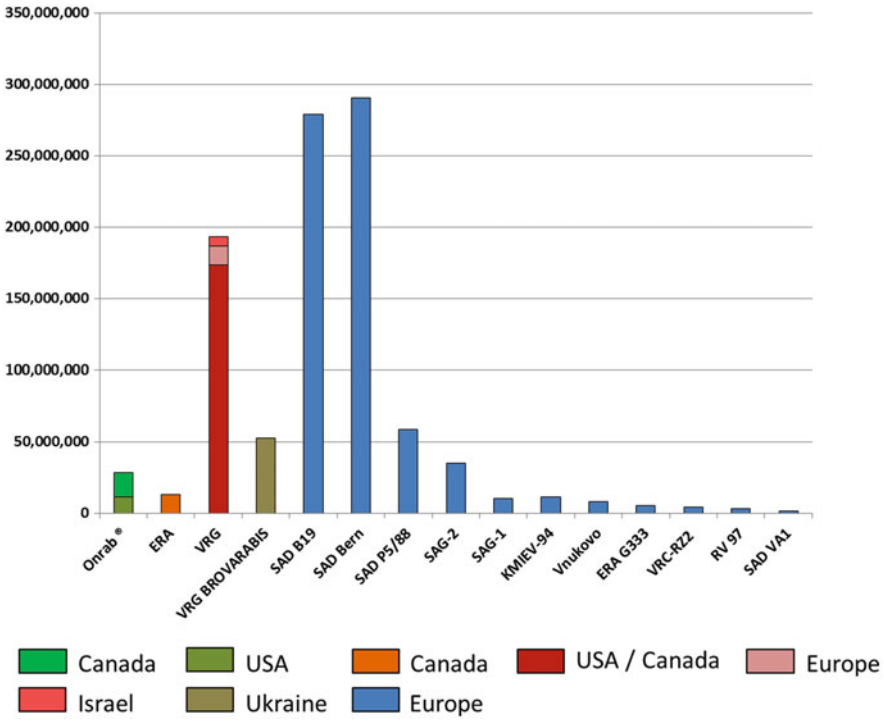

Fig. 2 Cumulative numbers of oral rabies vaccine baits distributed between 1978 and 2017 in Europe and North America. For Europe, numbers were extrapolated from the areas vaccinated as described elsewhere [85] and completed by datasets from the European rabies database

population and hence, had no epidemiological relevance [62, 86, 91]. Also, during the past four decades of ORV of wildlife in Europe and North America no adverse reactions or other critical incidents with any of the attenuated oral rabies virus vaccines after unintentional human contacts have been reported [62, 85].

Vector-Based Vaccines

Viral vectors were shown to not only tolerate the insertion of large foreign genes, but also to adequately display foreign antigens to the immune response in various disease models. Owing to advances in molecular technology, the insertion of the RABV gylcoprotein (G) became an option [92], particularly using pox viruses as a vector [93]. Thus, live recombinant vector-based vaccines could offer certain advantages for use in the control of rabies in wildlife, especially with respect to safety as rabies virus-associated disease cannot occur in vaccinated animals. The recombinant vaccinia virus Copenhagen strain expressing the ERA RABV G gene (V-RG) was the first oral rabies vaccine based on recombinant poxvirus [94]. This

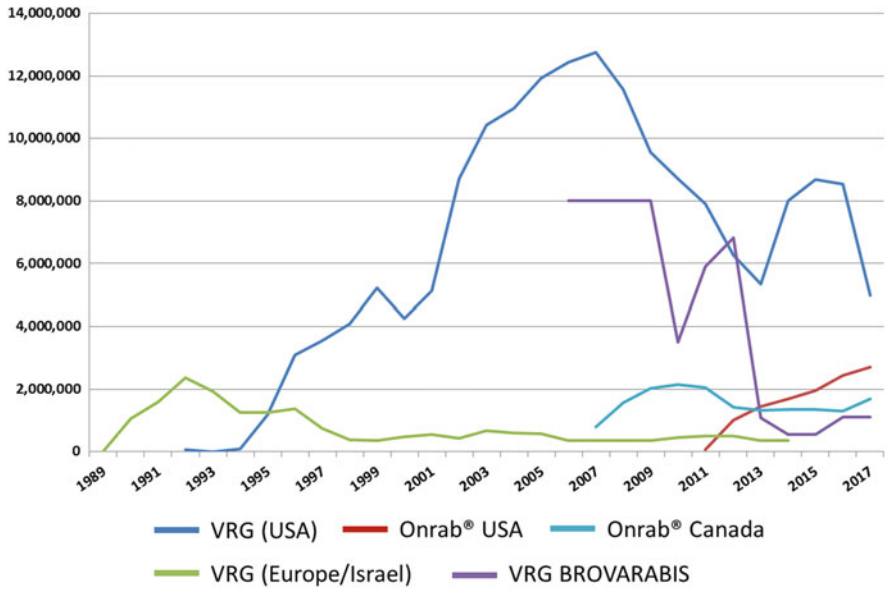

Fig. 3 Development of the number of vector-based oral rabies virus vaccines (V-RG and AdRG1.3) deployed in Europe and North America between 1989 and 2017. Figures for oral rabies vaccines used in non-European ORV programs were either retrieved from existing literature [95] or kindly provided by people mentioned in the acknowledgments

licensed vaccine has been widely used for the oral vaccination of raccoons, gray foxes and coyotes in North America, raccoons in Canada, jackals in Israel, and for red foxes in several Western European countries with more than 245 million V-RG baits distributed between 1989 and 2017 (Figs. 2 and 3; [95, 96]). Despite its efficacy in many hosts, V-RG does not induce adequate protective immunity in skunks [97], and appears to be less efficient in controlling raccoon rabies [98].

Alternatively, a replication-competent recombinant human adenovirus 5 expressing RABV G (AdRG1.3, [99]) as part of the vaccine bait ONRAB®, was capable of eliciting an immune response against rabies in target animals under laboratory [100–104] and field settings [98, 105–107]. During 2007 and 2017, about 28.5 million ONRAB® baits were distributed in Canada and the United States (Figs. 2 and 3).

While adverse events in animals were very limited [95] and reported to be restricted to lethargy, diarrhea, and vomiting [108], the exposure to V-RG has been associated with a severe skin inflammation and the possibility to cause systemic vaccinia virus infection in humans [108, 109]. Attempts to use the non-virulent Modified Vaccinia Ankara virus (MVA) as a backbone failed, as it was not able to induce an adequate immune response after oral administration [110].

Another disadvantage of vector vaccines is the potential interference by preexisting immunity against the vector, which may inhibit uptake of the recombinant and prevent the generation of sufficient anti-RABV immunity [111]. In fact, it

was shown that orthopox virus-specific antibodies were detectable in red foxes in Europe [112, 113], and raccoons from North America [114]. Against the background of substantial cross-reactivity between canine adenovirus type 1 (CAV-1) and CAV-2, the prevalence of antibodies against canine adenovirus in wild canids [115–118] preclude these vectors as potential vaccines for wildlife.

Over the past decades, various other vector viruses have been constructed for the expression of rabies virus genes (Table 2). Those viral vectors encompass several virus genera and families and are restricted to mostly promising proof-of-concept studies. To date, none except V-RG and ONRAB® have gone the long way of extensive testing to be then licensed for the prevention and control of wildlife rabies.

Reverse Genetics-Based Vaccines

Since the development of effective live-attenuated vaccines, next to new vaccine preparations utilizing well-established vectors, reverse genetics has continued to shape the domain of rabies vaccine discovery and development. Current RNA virus reverse genetics systems make use of multiple common features of RNA virus biology [1]. A common way to create a vaccine using reverse genetic techniques is to utilize plasmids to synthesize attenuated viruses. A great advantage of this technique is that it enables targeted alteration of virus genomes, i.e., deleting or inserting selected genome sequences and point mutations, by site-directed mutagenesis [1, 130].

For live attenuated RABV vaccines, safety is the foremost criterion [131]. According to recommendations of the World Health Organization (WHO), any oral rabies vaccine that can be used for immunization of wildlife must not cause disease in immunocompetent mice following intracerebral (i.c.) infection [72]. Consequently, in an attempt to further increase the safety profile or enhance the immunogenicity several new rabies vaccine virus constructs have been developed in recent years using reverse genetics (Fig. 1), also referred to as 3rd-generation oral rabies vaccines.

The great majority of oral rabies vaccine constructs generated using reverse genetics has emerged from the virus construct SAD L16, a cDNA clone of the oral rabies virus vaccine strain SAD B19 [132, 133] and resulted in one backbone named SPBN (the name is an acronym for the restriction enzymes *S*ma, *P*acI, *B*siWI, and *N*heI). Viruses based on this backbone still show residual pathogenicity, especially when administered intranasally [134]. Therefore, further modifications were necessary. The vaccine construct SPBN GAS, a SPBN GA-derived construct ($Arg_{333} \rightarrow GLu_{333}$), lacks the pseudogene (Ψ) and shows additional alterations in the RABV G where amino acid (aa) Asn_{194} is replaced by Ser [131]. Safety profiles could be further enhanced by insertion of additional one (SPBN GASGAS) and two (SPBN TriGAS) identical G genes containing the same genetic modifications [135, 136]. This overexpression of the RABV G results in the enhancement of apoptosis and antiviral immune response [137]. Overexpression of cytochrome c resulted in a strong increase in immunogenicity, coupled with the marked reduction

Table 2 Summary of vector virus constructs that could be eventually used as oral rabies vaccines for wildlife

Virus family	Genus	Vector virus species	Construct name	Immunogenic component	Target delivery	Target species	References
Poxviridae	*Parapoxvirus*	Parapoxvirus ovis (Orf virus)	ORFV121RABV-G	G-protein	i.m.	Cattle, swine	[119]
			D1701-V-RabG	G-protein	i.m.; other	Companion animals, others	[120]
	Orthopoxvirus	Raccoonpox virus	RCN-G	G-protein	i.m.; mucosal	Bats, other	[121]
Paramyxoviridae	*Avulavirus*	Avian avulavirus 1 (Newcastle disease virus)	rL-RVG	G-protein	i.m.	Dogs, cats	[122]
	Respirovirus	Human respirovirus 5 (Parainfluenza virus 5)	rPIV5-RV-G	G-protein	i.m.; i.n.; oral	Mice, other	[123]
Adenoviridae	*Mastadenovirus*	Human mastadenovirus C (Human adenovirus type 5)	Ad-0910G, Ad-0910N	G-protein, N-protein	Oral	Raccoon dog	[124]
			nrAd5-SRV9-G, nrAd5-Flury-LEP-G, nrAd5-BD06-G	G-protein	i.m.	Diverse	[125]
			HAd5-GB	G-protein + Flagellin	i.m., oral	Mice, other	[126]
			human Ad5 HCMV–intron–ERA	G-Protein	Oral	Mice, foxes, dogs	[127]
		Canine mastadenovirus A (Canine adenovirus 2)	CAV2-RVG	G-protein	Oral	Raccoons, skunks	[128]
			CAV-2-E3Δ-RGP	G-protein	Oral	Ferret badger, other wildlife	[129]

in pathogenicity, making the SAD SPBN-Cyto c(+) construct a candidate for a live rabies virus vaccine [138]. Today, other SAD backbones (BNSP) have been established [139, 140].

Site-directed mutagenesis was used to delete codons specifying the aa 176–181 of the RABV phosphoprotein (P). The resulting construct called SAD dIND activates interferon regulatory factor 3 (IRF-3) more efficiently thereby preventing interferon (IFN) type 1 induction [141]. The recombinant SAD rabies virus construct ORA-DPC encodes both, SAD and CVS G with an $Arg_{333} \rightarrow Asp_{333}$ exchange. In addition, this construct also possesses a 7-10 aa deletion in residues 143 to 149 or 139 to 149 encompassing a conserved LC8-interacting motif (K/RXTQT) in the P-protein [142, 143]. Expression of dendritic cell-activating molecules including the granulocyte macrophage colony-stimulating factor (GM-CSF) into the rabies virus was shown to enhance the innate and adaptive immune response to vaccination [144, 145]. Cloning of GM-CSF, bacterial flagellin and interleukin (IL)-15 into a recombinant attenuated derivate of the SAD B19 strain with two mutations in G protein resulted in the virus constructs LBNSE GM-CSF, LBNSE Flagellin, and LBNNSE IL-15, respectively, which are more immunogenic than the parent virus [144, 146].

Almost identical proof-of-concept approaches using site-directed mutagenesis were applied to generate recombinant oral rabies virus constructs in a recombinant ERA (rERA) backbone (Fig. 1). While the attenuated ERA333 [147], $rERAG_{333E}$ [148], and ERAG3G [149, 150] vaccine constructs have the same mutations at residue 333 of the G-protein as SAG2 [77], the genetic alterations in the recombinant RABV ERA GS [151, 152] are identical to those introduced in SPBN GAS ($Asn_{194} \rightarrow Ser_{194;}$ $Arg_{333} \rightarrow Glu_{333}$) [131]. It is highly questionable whether these essentially identical approaches can be considered as novel innovative contributions in the field of rabies vaccinology. An exception may be the ERA-N273/394-G333 construct that possesses mutations in the G and N protein of RABV. The construct was generated by combining the attenuating mutations at G333 and N273/394 ($Phe_{273} \rightarrow Leu_{273}$ and $Tyr_{394} \rightarrow His_{394}$) [153].

All in all, with the exception of ERA 333 [147] and SPBN GASGAS [154–159] none of these newly developed constructs with a higher safety profile have been tested in potential wildlife target and nontarget species in the frame of licensing.

Other Pipelines for Immunization of Wildlife

Besides live vaccines, the oral administration of purified RABV G derived from recombinant baculovirus infected cells induced protective immunity in raccoons [160], but this avenue was not further pursued perhaps because the economics of purification are generally not cost effective due to the low quantities of protective antigens produced. Another technology for the production and delivery of vaccine antigens is recombinant plant virus particles [161–163]. Several transgenic plants expressing the RABV G or plant virus-based rabies vaccines have been developed and were tested [164–166]. Other possibilities to vaccinate animals by the oral route

are the development of live attenuated bacteria like *Salmonella*, *Shigella*, and *Listeria* expressing foreign antigens as vaccines [167]. Although the use of live bacterial-vectored vaccines has certain advantages, e.g., relatively easy to manufacture at low costs and safety advantages, and despite the development of several bacterial vectors for the oral delivery of vaccines [168], it has not yet been attempted for rabies.

DNA vaccines use the DNA sequence coding for the desired antigen, which is inserted into bacterial plasmids under the regulation of an eukaryotic promoter in such a way that it can be transcribed into mRNA. The purified plasmid DNA is then inoculated directly into the host where it can transfect cells. Once in the nucleus of the hosts' cell the mRNA is translated into the encoded gene and subsequently presented to the immune system [169].

Thus far, although numerous studies have demonstrated the relative effectiveness of DNA-based rabies vaccines at inducing RABV-specific VNA and protection [170–173], there is hardly progress beyond proof-of-concept studies. As only oral vaccination is feasible for wildlife, the fact that plasmid DNA can be packaged in vitro into a virus-like particle (VLP), which elicit an immune response also after oral application appears promising [174].

Legal Basis and Requirements for Oral Rabies Vaccines for Wildlife

A general framework for veterinary vaccine manufacturing and the respective requirements for oral rabies vaccines for wildlife is provided by the World Organization for Animal Health [175]. In fact, efficacy and safety criteria for oral rabies vaccines were initially discussed and defined through the Veterinary Public Health Department of WHO [15, 72, 176]. Guidelines for marketing authorization and/or licensing of oral rabies vaccines have also been laid down in official requirements by regulatory authorities, i.e., by the European Medical Agency [177], and the United States Department of Agriculture (USDA), under Title 9, Code of Federal Regulations [178]. In order to minimize the need to perform separate or additional studies for regulatory authorities of different countries in the frame of marketing authorization and/or licensing of vaccines, an International Cooperation on Harmonization of Technical Requirements for the Registration of Veterinary Medicinal Products (VICH) was established ([179] (https://www.vichsec.org/)). To date, the European Union, the United States, and Japan are participating in the VICH and negotiations on harmonization are ongoing.

Studies done under laboratory conditions should be performed and managed in accordance with the principles of Good Laboratory Practices (GLP), while field safety studies should be conducted in conformity with the principles of VICH Good Clinical Practices (GCP) [180]. The same applies to the manufacturing process of the respective commercial product which also has to be under GMP(-like) conditions.

Principally, oral vaccines for wildlife should demonstrate their immunogenicity and efficacy in the respective target species [177, 178]. For efficacy tests in

vaccinated animals, at least 25 animals shall be used as vaccinates. Criteria for acceptance in challenge tests shall be death due to rabies in at least 80% of the control animals while at least 22 of 25, 26 of 30, or a statistically equivalent number of the vaccinates (if a higher number of vaccinates was used) should survive a period of 90 days after a virulent rabies virus challenge [181]. There are still some deviations, e.g., while in the European monograph the time point of challenge is 180 days post vaccination [177], the requirements for licensure in the United States are a challenge after 365 days post vaccination [178].

Because of the nature of the product as a replication-competent live virus, the safety of the vaccines is of high importance and should be assessed in target and nontarget species. The special concern for nontarget species is unique for oral rabies vaccines as baits are distributed unsupervised and can be located and consumed by any animal interested. This makes it very difficult to lay down in which species the vaccine should be tested as the vaccine is used in many different habitats inhabited by many different animal species. Additionally, for recombinant vaccines, a risk assessment for risks to animals, humans, and the environment should be undertaken prior to the use in the field.

As the immunogenicity and efficacy is directly influenced by the vaccine virus titer, a minimum effective titer should be established before marketing release, representing the lowest titer of vaccine that can protect 100% of the target experimental animals against a virulent rabies challenge. To compensate for titer losses after distribution in the environment the batch-release titer in the vaccine bait should be above the minimum effective titer.

Another influential component of any vaccine for oral use is the bait consistency. The bait matrix should remain thermostable under field conditions for days, and the stability is assessed if it remains in its original shape [175].

Discussion

Without any doubt, oral rabies vaccines have gained great reputation in controlling rabies in wildlife in the Northern hemisphere [26]. During the past 40 years, those vaccines in combination with a well-defined vaccination strategy formed the basis for the elimination of red fox-mediated rabies in vast areas of Western and Central Europe and North America [28, 85, 182]. While until about 2011, ten European countries self-declared freedom from rabies due to long-term and large-scale implementation of ORV programs [183], in recent years, Estonia (2013), Latvia (2015), and Slovenia (2016) joined the ranks of European rabies free countries. Three additional countries including Slovakia, Lithuania, and Croatia are meeting criteria for a rabies free status according to OIE definitions (http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_rabies.htm).

While in North America, vector-based oral rabies vaccines were essential in eliminating rabies in coyotes and gray foxes [27, 29, 30], they still play an important role in containing raccoon- and skunk-mediated rabies [27].

However, there are still hurdles ahead, e.g., practically, scientifically, and from regulatory standpoints. Practically, the best vaccine construct does not work if the associated bait is not readily accepted by the target species. In this respect, attempts were made to modify standard baits so that the baiting success for species other than foxes is increased [184–189]. The recent emergence of rabies in the Formosan badger on the Taiwan Island and bait trials with various formulations indicate the difficulties when using a bait developed and optimized for one particular target species for other species [189]. Besides more efficacious oral rabies vaccines and species-specific baits, the development of effective bait delivery and vaccination strategies for these reservoir species remains a difficult task.

Surprisingly, after decades of ORV the precise mechanisms by which oral vaccines elicit an immune response are still poorly understood. Particular challenges are differences in vaccine titers needed to induce a protective immune response against rabies after ORV in different reservoir species, in particular raccoons and skunks [104, 128, 190–194]. Studies in red foxes and skunks demonstrate that the palatine tonsils play a critical role in vaccine virus uptake. The absence of virus-infected cells in palatine tonsils of skunks suggests a less efficient uptake of or infection by vaccine virus, which may lead to a lower responsiveness to oral vaccination [195]. Further studies are needed to elucidate the mechanisms in those species that appear rather refractory to oral vaccination.

The initial objective for safer and/or more immunogenic vaccines is somewhat contradicted by regulatory hurdles, for both the licensure and the actual application in the field. For example, while vector-based oral rabies vaccines and oral rabies vaccines based on reverse genetics both are genetically modified organisms, their use, is often controversially discussed irrespective of their safety profile. Risk assessments need to value inserted foreign sequences/transgenes, their ability for reconversion, recombination, and dissemination in the population and the environment [196, 197]. The same applies to plant-based vaccines, which were able to protect sheep after oral feeding against a rabies challenge [198].

Because rabies is a fatal disease, for oral live attenuated rabies vaccines safety for target and nontarget populations (humans and competitive species) is of utmost importance. In fact, in one respect or another none of the 1st- and 2nd-generation- and vector-based oral rabies vaccines completely meet the requirements for oral rabies vaccines as regards avirulence for target and nontarget species, genetic stability, and no excretion of live virus [80, 81, 181, 199]. To this end, different approaches have been applied (as described in this chapter) to develop highly attenuated rabies virus vaccines for oral vaccination of mesocarnivores [200]. In this respect, while safety of one 3rd-generation oral rabies vaccine has been demonstrated [156–158] those of other candidate vaccines still have to be assessed. In any case, determining and refining the balance between safety and efficacy is a delicate matter [200], as increased attenuation or over-attenuation will run the risk of losing immunogenicity due to reduced replicative fitness of the vaccine virus [201].

The pioneering work of the development of a vaccine for wildlife has also helped in conservation aspects. Rabies is not only an important public and veterinary health burden, but it can also threaten endangered wildlife species [202], such as the golden

palm civet (*Paradoxurus zeylonensis*) [43], the African wild dog (*Lyacon pictus*) [203, 204], and the Ethiopian wolf (*Canis simensis*) [205]. For the latter two species, the objective of wildlife rabies vaccination programs was not necessary to eliminate infection but to protect these highly endangered species [206–208].

Free roaming, stray, and feral dogs also represent a challenge to rabies control using parenteral vaccination. To this end, similar to wildlife ORV may help in increasing the herd immunity in those dogs to a level at which the transmission cycle of rabies is disrupted.

Acknowledgments The authors would like to thank Patrick Wysocky for excellent technical support. Gratefully acknowledged is the contribution of data on oral rabies vaccine doses distributed in the United States, Canada, and Israel by Richard Chipman, Kathy Nelson, Tore Buchanan, Christine Fehlner-Gardiner, Alex Beath, and Boris Jakobson. We also thank Adriaan Vos for valuable comments.

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Abstract

Human rabies vaccines have changed dramatically since Pasteur and his colleagues produced and administered a crude mixture of infected rabbit spinal cord to two severely bitten young boys in 1881. The evolution in the development of rabies vaccines has improved the efficacy and safety of treatment but human rabies vaccines are still costly and unavailable to many patients living at risk in poor regions of the world. Human rabies vaccines are produced from a number of different cell culture substrates and seed viruses and all are required to have a potency of 2.5 IU per intramuscular dose. The Biological Standardisation Program is currently overseeing an expert working group evaluating a feasible in vitro replacement for the National Institute of Health (NIH) in vivo potency assay. The current human cell culture rabies vaccines are highly efficacious, generally well-tolerated and safe. In spite of the presence of accelerated, economical intradermal regimen for pre- and postexposure prophylaxis completed in 7 days, the vaccine needs to be refrigerated [1, 2]. A handful of new rabies biologicals are currently in the pipeline; however, very few human rabies vaccines have reached clinical trial phase. Rabies is not a disease that can be eradicated and therefore there will continue to be a need for less costly, more accessible and thermostable vaccines for human use.

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H. C. J. Ertl (ed.), *Rabies and Rabies Vaccines*,
https://doi.org/10.1007/978-3-030-21084-7_4

Introduction

Rabies has the highest mortality rate of any infectious disease known to mankind with over 98% of all human deaths being attributed to exposure to infected dogs [3–5]. Once clinical signs are present, death is almost invariably the final outcome [1, 6–8]. If it were not for the fact that disease can be prevented even after exposure to virus, rabies would most likely rank highest on the most dangerous list of infectious agents. Fortunately for the hundreds of thousands of patients exposed to rabies annually throughout the world, the onset of rabies can be prevented through appropriate wound care after an exposure has occurred and the administration of highly effective anti-rabies biologicals including rabies immune globulins (RIG) and cell culture rabies vaccines (CCVs) [5]. This characteristic of rabies is attributed to the fact that the reproduction process of virus in a newly infected patient is almost always slow enough to allow the prompt administration of rabies vaccine to elicit a protective immune response and thus prevent the onset of clinical rabies [1, 9, 10]. In addition, prompt and appropriate cleaning of all open wounds and administration of RIG helps to inactivate viruses that may have entered muscle tissue during exposure [5, 11].

Development of Human Rabies Vaccines

Although the initial reports of how rabies was transmitted from infected to uninfected animals were reported in the early nineteenth century, it was Louis Pasteur and his colleagues Roux, Chamberland, and Thuillier who developed the first effective, albeit crude, human rabies vaccine [12]. Development of the first human rabies vaccine resulted from Roux's observation that by drying the spinal cords of infected rabbits for various lengths of time, rabies virus could be attenuated with full attenuation occurring after 15 days. Based on Roux's discovery, Pasteur began experiments by vaccinating dogs with rabies-infected spinal cord that had been dried for various lengths of time beginning with the most attenuated and, presumably, noninfectious tissue. Although the results of these first experiments were nonconclusive, Pasteur elected to vaccinate two severely exposed human patients, Joseph Meister and Jean Baptiste Jupille, based on his initial findings [12]. These two patients had been severely bitten by rabid dogs and most likely would have contracted rabies without the intervention of Pasteur's crude vaccine. Both patients did survive and Joseph Meister went on to be the gatekeeper at Pasteur's laboratory [13]. Pasteur's decision to treat these two human patients based on the limited data that he had available would be completely unethical in today's world of modern medicine and was highly criticized by the medical community when Pasteur made the decision to administer his rabbit spinal cord vaccine in 1881. However, it is important to remember that there was no effective treatment against rabies after an exposure and these two patients would almost certainly have died without intervention.

Nerve tissue rabies vaccines (NTV) produced from sheep or goat brain replaced the rabbit spinal cord Pasteur vaccine and was in use until the mid-nineteenth century when the development of more purified rabies vaccines began to occur [14]. NTV produced from sheep brain vaccine is still being produced and administered to humans in Ethiopia [15, 16]. In the search for a less reactogenic rabies vaccine, Fuenzalida and colleagues from Latin America improved the production process by using suckling mouse brain tissue in place of adult sheep or goat brain tissue [17, 18]. This change in animal tissue eliminated the presence of myelin in the vaccine and thus helped to reduce adverse reactions caused by the vaccine [19, 20]. However, although the incidence of reported adverse reactions was reduced when Fuenzalida vaccines replaced cruder sheep brain vaccines, when reactions did occur they were more severe [14]. Fuenzalida type human rabies vaccines are currently still being used in Bolivia and Algeria [21, 22].

A shift from production of rabies vaccines in animal brain tissue occurred in the 1930s when virologists developed improved methodologies for producing viruses through inoculation of embryonated eggs. This discovery led to the development of the first duck embryo vaccines (DEV), no longer in use today. These first DEV are considered crude compared to the CCVs that are available today, but they provided a safer and more purified and uniform vaccine. Although the first DEV was a gigantic step forward in improving the effectiveness of human rabies vaccines, they were less potent than desired [10, 23]. Access to advanced technologies aimed at improving the purity, potency, and uniformity of vaccines led to the development and marketing of highly purified avian based duck embryo vaccine (PDEV) [24, 25]. The production process for modern avian based vaccines enables manufacturers to remove virtually all egg-based proteins and myelin, thus providing a highly purified product. Highly purified avian-based human rabies vaccines have been used for over three decades with well-documented safety and efficacy.

The adaption of primary cell lines to grow in vitro was a breakthrough in the production of viral vaccines [26]. There have been a number of different cell substrates that have been employed in the production of viral vaccines since Pasteur and his colleagues dried the first rabbit cord in an attempt to find a prevention for the onset of rabies (Table 1). Primary cells are used to produce one of the two types of human rabies vaccine that have received WHO prequalification [27, 28]. The use of primary cells as a substrate for viral vaccines has both advantages and disadvantages. Primary cells are nontumorogenic and thus must be consistently replaced and are not suitable for use in continuous cell line production of vaccines [10, 29]. The fact that the cell culture must be replenished increases the opportunity for contamination that may not be so evident in closed continuous cell culture production systems. Embryonated eggs used for the production of rabies virus vaccines must be certified Specific Pathogen Free (SPF). This fact, as well as numerous quality assurance and good manufacturing practices, help ensuring that contamination issues are eliminated. Purified Chick Embryo Cell Rabies Vaccine (PCECV) is produced using primary cell culture technology and has a well-documented history as a safe, effective, and well-tolerated vaccine [30–33].

Table 1 Historical development of human rabies vaccines

Substrate	Inactivation process	Name of vaccine
Nerve tissue vaccine		
Rabbit spinal cord	Dried over a series of days	Pasteur treatment
Sheep, goat, or rabbit brain	Phenol	Fermi
Sheep, goat, or rabbit brain	Phenol	Semple
Suckling mouse brain	β-Propriolactone	Fuenzalida
Avian		
Duck Embryo	Formalin	Duck Embryo Cell Rabies Vaccine (DEV)
Purified duck embryo	β-Propriolactone	Purified Duck Embryo Cell Rabies Vaccine (PDEV)
Cell culture		
Human fibroblasts	β-Propriolactone	Human diploid cell rabies vaccine (HDCV)
Fetal rhesus cells	β-Propriolactone	Rabies Vaccine Absorbed (RVA) CHECK THIS
Primary Syrian hamster kidney cells	Formalin	Primary hamster kidney cell rabies vaccine (PHKCV)
Chick embryo cells	β-Propriolactone	Purified chick embryo cell rabies vaccine (PCECV)
Vero cells	β-Propriolactone	Purified Vero cell rabies vaccine (PVRV)

Adapted from Rupprecht CE, Nagarajan T, Ertl, H. Plotkin's vaccines: cell culture rabies vaccines. Philadelphia (PA): Elsevier; 2018. p. 927

Human rabies vaccine produced using diploid cells is still in use today. They are highly immunogenic and have a well-documented history of safety [34–36]. Human diploid cell rabies vaccine (HDCV) can be used in a semicontinuous production system that allows multiple expansions of materials [10]. The first HDCV was developed at the Wistar Institute and documented evidence indicates that they are safer and more immunogenic than primary hamster kidney cell rabies vaccine (PHKV) [10]. The production costs of HDCV limit its use in resource-poor countries.

Continuous cell lines, for example, Vero cells, provide the advantage of being able to maintain a closed system for the production of viral vaccines. They have an infinite life span and published studies have indicated that they are nontumorogenic for multiple passages [37]. Purified Vero cell rabies vaccine (PVRV) was developed and marketed in the 1980s and has proven to be a highly efficacious, safe and well-tolerated vaccine [38–40]. PVRV produced by Sanofi Pasteur has met WHO Prequalification standards and is distributed globally. The development and global distribution of PVRV and PCECV have enabled the lives of millions of patients exposed to rabies to be saved.

Registration of Human Rabies Vaccines

As described above, rabies vaccines for use in humans have dramatically improved in purity, efficacy, and reliability since Pasteur and his colleagues produced the first NTV in the late nineteenth century. The World Health Organization (WHO) has published standard recommendations for the production of human rabies vaccines and has strongly recommended that NTVs, manufactured in vivo by inoculating and subsequently harvesting the brain of infected animals, be replaced by vaccines produced in vitro from cell culture [5]. Although rabies vaccines produced for use in animals may be formulated as adjuvanted, modified-live, recombinant, or inactivated, the only CCVs currently produced and licensed for use in humans are inactivated vaccines. An extensive review detailing the manufacturing process of rabies vaccines has been published elsewhere [41].

It is the responsibility of national governments to approve, license, and monitor the use of human rabies vaccines within their country. Working with global experts in the field of vaccinology and standardization, WHO has established a set of criteria for production of biologicals, including rabies vaccines. Information on specific production recommendations are freely available on the WHO website [27]. Additionally, vaccine production facilities that have been evaluated and have met the WHO requirements for good manufacturing processes can apply for WHO Pre-qualification of a vaccine, an indication that their vaccine production meets or exceeds the WHO requirements for production and human use [27].

WHO recommends that every intramuscular dose of CCV contain a minimum of 2.5 IU of antigen as measured by the NIH potency test [5, 19, 42]. There is no additional potency requirement recommended by the WHO for intradermal administration of rabies vaccines [42]. Published data have shown that vaccine containing 2.5 IU per intramuscular dose will produce an adequate immune response when administered intramuscularly or intradermally at a volume of 0.1 IU using the Thai Red Cross PEP ID regimen [43, 44]. However, individual governments may have specific potency requirements that exceed the WHO required level of 2.5 IU per intramuscular vial for use within their own country.

It is clear that the potency of human rabies vaccines will vary from batch to batch, irrespective of what brand of vaccine is being produced. This relates to the fact that the production of CCV involves biological systems as well as a number of different steps in the manufacturing process. All of these processes will influence the amount and stability of viral glycoprotein (G protein), and thus the potency, in the final product. Therefore, in order to ensure that every batch of human rabies vaccine has sufficient antigenic properties to elicit an adequate immune response after vaccination using one of the WHO recommended regimens, strict quality control is required [45]. The NIH mouse inoculation assay is currently the potency test accepted and required internationally for evaluating the potency of human rabies vaccines. The NIH test for potency was adopted by WHO over 50 years ago [10, 46, 47]. For the past several years, there has been an ongoing effort headed by International Regulatory Agencies, WHO, global experts, and the pharmaceutical industry to replace the NIH test for potency with a more humane, accurate, and sensitive assay [48–51]. There are

many inherent problems with the NIH test, undoubtedly one of the most concerning reasons to replace the NIH test is that it requires large numbers of animals to be sacrificed as part of the assay.

In 2011, several international agencies from at least six different countries including regulatory agencies, international and national health programs, statisticians, and pharmaceutical companies manufacturing human rabies vaccines held a workshop to find a suitable replacement for the NIH potency assay [52]. The workshop named the International Coordination Committee on the Validation of Alternative Methods (ICCVAM) agreed that an alternative to the NIH potency assay was feasible and desirable. The objective behind the initiative was to improve compliance with the 3 R's, that is replacement, refinement, and reduction in the use of animals in research; to reduce batch testing costs and thus shorten lead times to batch release for medical use and help to reduce vaccine shortages [42, 53]. Many studies evaluating replacement assays for the NIH test have been conducted and most if not all rabies vaccine manufacturing facilities have already replaced the use of the NIH test for their in-line assays to evaluate potency during the manufacturing process [52, 54, 55]. Considering that it is an accepted fact that protection against rabies is due to the production of neutralizing antibodies targeted against the native membrane-associated trimeric form of the G-protein, the ICCVAM agreed that replacement of the in vivo NIH potency test with a completely in vitro assay is an achievable goal [56–58]. Additionally, the European Pharmacopoeia recommends the use of validated serological or immunochemical assays as a replacement for the NIH potency test for rabies vaccines [52].

Five laboratories participated in a study to evaluate a standardized sandwich ELISA using several different reagents and assay designs. It was agreed that the next step would be to undergo formal validation of the selected assay under the auspices of the Biological Standardisation Program (BSP) of the European Directorate for the Quality of Medicines & Healthcare/Council of Europe and European Commission. In 2017, the working group published the results of their study and have established the next steps: (1) To conduct an international collaborative study overseen by the Biological Standardisation Programme (BSP) that will evaluate repeatability and reproducibility using the two monoclonal antibodies selected from the previous studies; and (2) Upon completion of the collaborative study, the agreed upon ELISA will be introduced as an alternative to the NIH test for potency of human rabies vaccines [52]. It is expected that the finalization of the replacement of the NIH test will occur within the next few years.

Evaluation of Rabies Vaccines

Considering the millions of patients exposed to rabies who have received CCVs since they were initially developed and marketed over three decades ago and the extremely few documented cases of human rabies cases after vaccination, it is evident that there are very few "true" rabies vaccine failures [59–61]. When human rabies vaccines are administered according to the recommendations specified

by WHO, they are among the most efficacious human vaccines available. Almost every case of human rabies that has been reported after an exposure has occurred can be attributed to the patient not seeking PEP; a delay in seeking PEP; or nonadherence to one of the WHO recommended PEP regimens and has not been attributed to a lack of vaccine efficacy. Direct evidence of the efficacy of CCVs, administered either intramuscularly or intradermally, in conjunction with RIG, has been documented in many published scientific papers [38, 62–65]. Indirect evidence documenting the immunogenicity of rabies vaccines has also been published in numerous serological clinical studies [32, 39, 40, 66]. Accumulated data from the administration of CCVs over the past three decades have proven that they are safe, immunogenic, and long-lasting and should never be withheld from anyone that has been exposed to rabies [40, 67–69].

There are several criteria by which new human rabies vaccines are evaluated during the initial development stage, during clinical development, and post-marketing surveillance. These include but may not be limited to: antigenic potency; efficacy; immunogenicity; safety; and tolerance. Evaluating the potency of human rabies vaccines has been discussed above and WHO recommends a potency of 2.5 IU/ intramuscular dose of human rabies vaccine. However, the ultimate objective of any vaccine is to provide protection against disease. Historically, evaluation of the efficacy of a CCV has involved conducting clinical trials using good clinical practices (GCP) procedures in approximately 100 patients documenting survival for at least one year after an exposure to a laboratory-confirmed rabid animal has occurred [31, 62, 70]. Conducting clinical trials to provide efficacy data has been a necessary requirement when new vaccine technologies are being considered as replacements for rabies vaccines that are currently being used to prevent rabies. The efficacy data published since CCVs were developed and marketed has provided sufficient assurance that these vaccines are life-saving biologicals.

As mentioned above, conducting clinical trials under GCP conditions to prove vaccine efficacy is expensive, difficult and time consuming, and putting exposed patients at risk with unproven vaccines is unethical. Scientific evidence indicates that neutralizing antibodies are critical for protection against rabies and therefore, measuring the presence of neutralizing antibodies after rabies vaccination continues to be a very useful tool for assessing the effectiveness of a CCV [52, 56]. There are two assays recognized by WHO to measure neutralizing antibodies to rabies virus, specifically the Rapid Fluorescent Focus Inhibition Test (RFFIT) and the Fluorescent Antibody Virus Neutralization Test (FAVN). Both assays are complex to perform and require specific laboratory equipment as well as trained personnel. Research on measuring the level of neutralizing antibody titers after vaccination with CCVs produced from different seed viruses has indicated that the amount of neutralizing antibody that can be measured within the first 14 days is dependent upon the degree of homogeneity between the seed virus used in the production of vaccine administered and the challenge virus used in the neutralizing assay [71, 72].

The development of CCVs dramatically improved the level of safety and tolerance of rabies vaccines for patients that need PEP. There continue to be a handful of reported adverse events associated with the administration of CCVs but PEP should

never be withheld from any patient that has been exposed to rabies. Mild systemic adverse events are often reported in association with the administration of CCVs and include pain at the injection site; erythema; swelling; fever; chills; and headache malaise [24, 30, 31]. In the case that a patient receiving CCV does experience a serious adverse event, medical advice should be sought immediately [73].

Pipeline for Human Rabies Vaccines of the Future

CCVs were labeled as "modern" when they were first developed and marketed 40 years ago and have dramatically improved the safety and effectiveness of human rabies vaccines. Unfortunately, they continue to be expensive for the majority of people living at daily risk of infection. This is a result of several factors including the fact that rabies vaccines are often not available in rural clinics in rabies endemic countries; they require multiple doses over an extended period of time thus requiring multiple clinic visits often necessitating expensive travel expenses; and current vaccines require refrigeration that is often not reliable in regions with limited infrastructure [4, 74]. These, somehow have been improved by introduction of a "one-week program" for both PreP and PPP [1, 2]. Considering the fact that the technology required to develop effective rabies vaccines that could overcome these barriers and thus improve accessibility is available, it is an attainable goal to encourage the development of a new generation of human rabies vaccines.

There are a handful of new rabies biologicals, including rabies vaccine, in the initial stages of development, most of the vaccines under development are focusing on animal use [10]. Recently, Kalimuddin et al. [75] published promising data from a phase II clinical trial examining the immunogenic properties of an adjuvanted human rabies vaccine. The adjuvant that they used was a refined form of Polyinosinic-Polycytidylic acid stabilized with kanamycin and calcium (PIKA). Initial animal studies using this adjuvanted vaccine showed high titers in hamsters and survival from challenge. The phase II study in humans used the 2-1-1 Zagreb regimen [76] and reported 57.6% of the subjects who received the PIKA rabies vaccine had titers of 0.5 IU by day 7 compared to 43.8% of the control group that received a nonadjuvanted CCV.

Nanoparticle vaccine technology based on a baculovirus-derived glycoprotein is a RNA-active rabies vaccine that is being tested in animal models but whether this research will lead to new rabies vaccines for humans remains to be seen [77, 78]. Other types of novel rabies vaccines include an E1-deleted adenovirus vector or chimpanzee-origin expressing the rabies G-protein and they may provide future hope for a less costly human rabies vaccine [79].

In conclusion, although the currently available CCVs are highly efficacious, they are expensive and this contributes to the fact that they are inaccessible in economically deprived regions of the world. CCVs presently also require multiple doses to ensure protection after exposure, again contributing to the cost for PEP. Despite the fact that the WHO has been urging the replacement of NTVs for the past two decades, three countries still produce and administer these older and less purified

vaccines to patients seeking PEP. The incentive for manufactures to invest in other pharmaceuticals related to noninfectious diseases continues to deter the further development of human rabies vaccines that could provide an alternative to multiple doses and also provide longer-lasting thermostability. The goal to reach a human "rabies-free" world within the next decade is a worthwhile goal and one that is attainable but it will depend on the extended availability of effective and less expensive human rabies vaccines.

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Rabies Vaccines, Prophylactic, Peru: Massive Rabies Pre-exposure Prophylaxis for High-Risk Populations

Sergio E. Recuenco

Abstract

Since 1975, Peru has reported human rabies outbreaks due to vampire bat transmission, among populations living in their Amazon Basin region. Responses with traditional rabies control tools, rabies postexposure prophylaxis for bitten individuals with suckling mouse brain vaccine (SMBV) and bat culling, failed to stop human deaths, which mainly were seen in children from native populations.

Human outbreaks increased significantly from 2007 to 2010, and by 2011, Peru started a massive rabies pre-exposure prophylaxis (PreP) program targeted to populations living in high-risk areas for vampire bat rabies. Covering parts of the Peruvian Amazon rainforest region, the program started in Condorcanqui Province, the source of most of the human rabies cases. The country's rabies prevention policy put in place for the PreP program mandate the exclusive use of cultured cell vaccines, including human diploid cell vaccine (HDCV), and purified Vero cell vaccine (PVCV).

The campaign was progressively expanded from 5 provinces in 2011 to 20 provinces in 2016, and successfully prevented new human rabies cases, with zero reports among vaccinated population, while rabies virus circulation and bat bites to people is still ongoing.

Massive Rabies PreP implemented as a national rabies prevention policy has no precedent and the Peruvian experience provides insights for further use of this strategy for other high-risk areas for rabies exposures.

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H. C. J. Ertl (ed.), *Rabies and Rabies Vaccines*,
https://doi.org/10.1007/978-3-030-21084-7_5

Introduction

Vaccination is the cornerstone of rabies prophylaxis and prevention. Since the original Pasteur's rabies vaccine in 1885, rabies prophylaxis by vaccination became the only effective intervention to avoid rabies clinical onset in an exposed individual, typically a child bitten by a rabid dog [1–3]. The success of rabies vaccination led to the development of rabies prevention activities and control strategies, such as vaccine design and production for humans and animals, rabies laboratory diagnostic methods, rabies surveillance, and massive canine vaccination. All of those activities involved a level of risk for rabies exposure for scientists and workers, whom could be infected with rabies by manipulating infective matter containing rabies virus, or by interacting with potentially rabid animals and getting in contact with infective saliva of a dog or wildlife. The need for rabies prophylaxis due to occupational risk to rabies exposure was defined and schedules for rabies vaccination targeting people not yet exposed, but with a high likelihood for near future exposures were developed. Such a strategy is known as Pre-Exposure Prophylaxis (PreP) and is part of standard rabies prevention recommendations [4]. Although occupational risk is a clear indication for PreP, it is neglected in some exposed workers, as documented in recent studies [5, 6].

The indication for PreP was extended to include travelers going to areas considered of high risk for rabies [7–9]. Current standard travel health advice, such in the Centers for Disease Control (CDC) Yellow Book, lists places of risk for rabies [10]. While the risk for travelers is temporary, the risk for populations living in high-risk areas for rabies is assumed to be constant. The indication to provide PreP for populations living in those areas was not included in the World Health Organization (WHO) PreP recommendations until recently [11]. A first WHO suggestion for massive PreP consideration for wildlife rabies prevention was declared in 2009, when the risk particular in Amazonia for vampire bat bites rabies was acknowledged as worthy to develop specific local rabies prevention policies [12]. Despite those recommendations, no policies were developed in any country to introduce massive PreP vaccination. In the meantime, with extended intervention of massive canine rabies vaccination, dog-transmitted rabies was eliminated from developed countries and largely controlled in Latin America. With canine rabies control, wildlife rabies seemed to emerge or became more readily detected, including rabies transmitted by bats.

Vampire Bat Rabies as a Risk to Indicate for PreP

In North America, rabies circulates in non-hematophagous bats and other wildlife causing human deaths every year. Cases are isolated and sporadic, with a low frequency of exposures among the population, making it unnecessary to consider massive interventions targeting large populations [13]. Hematophagous bats or vampire bats are only present in Latin America, from Mexico to the North of the Argentine Republic. From three known bat species feeding on blood, the most

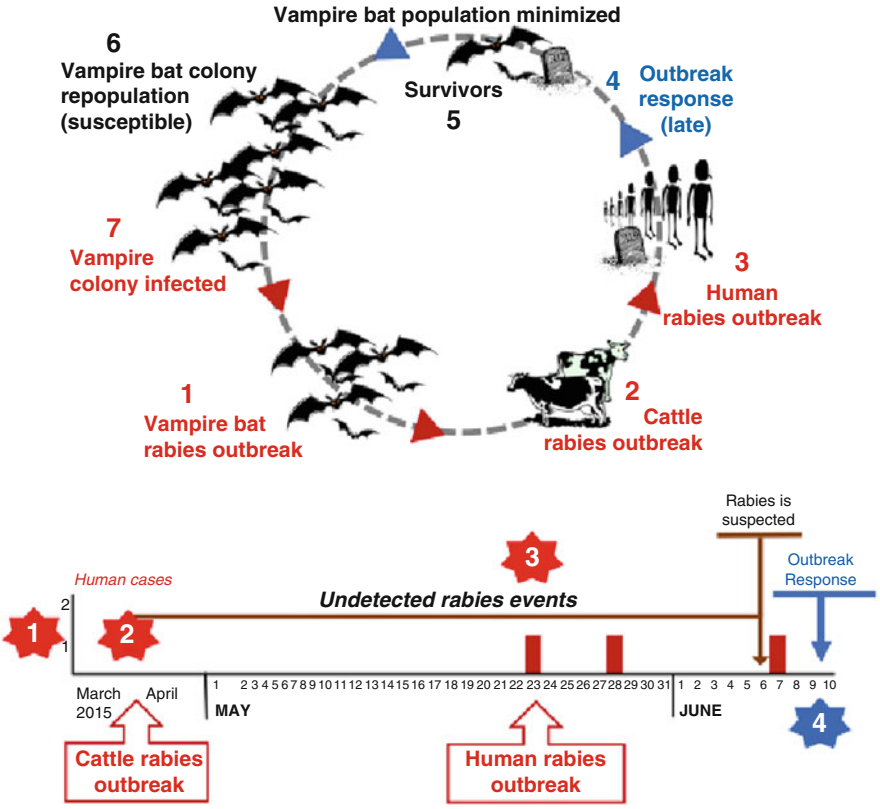

Fig. 1 Sequence and relationship between vampire bat rabies outbreaks in cattle and humans. The timeline compares a real outbreak from Soledad, Peru in 2015 with the typical sequence of event for vampire bat rabies in endemic areas. Source of outbreak data: Peru Ministry of Health

important for rabies transmission is the common vampire bat, *Desmodus rotundus*, ubiquitous in all Latin American countries with the exception of Chile [14]. Vampire bat attacks of cattle have been observed in Peru since the end of the eighteenth century [15]. The role of vampire bats as vectors for rabies in cattle was demonstrated by Pawan in 1936 [16, 17]. *D. rotundus* prefers to feed on bovines, horses, or pigs, but can feed on chicken and other wildlife mammals [18–21]. Although humans do not seem to be the preferred food source for *D. rotundus*, vampire bites to humans occur in variable frequency across the Amazon Basin [22, 23]. Reports vary from 44 to 88% recently bitten individuals in a given population. The highest reported frequency was in Datem del Marañon in Peru, a province adjacent to the vampire bat rabies endemic province of Condorcanqui, where a survey in 2010 counted 100% of homes where at least one member of the family had been bitten in the recent 6 months [24].

The sequence of events in a typical human rabies outbreak due to vampire bat rabies is well documented [23, 25–28]. Figure 1 presents the identifiable series of

events that are part of a cycle allowing persistence in endemic areas. The cycle starts with a colony of susceptible vampire bats, getting infected likely from contact with individuals from a neighboring colony. Recent studies indicate that outbreaks of rabies in vampire bat colonies occur in waves, in concordance with observations of several sequential rabies outbreaks of cattle and humans in an adjacent district [29]. Rabid vampire bats can continue feeding on their usual cattle or other preferred prey transmitting rabies to the bitten animal before neurological symptoms make the infected bat unable to fly. Several bats can bite one cattle, horse, or other animals large enough to sustain multiple bites. Some studies suggest that vampire bats are loyal to their food source, meaning they would repeatedly use the same food source as long as it is still available [30] (AG, unpublished data). Population of bats is reduced due to rabies. Surviving vampires will be immune and not contagious, and the following months and years will allow for recovery of the colony's population, until a number of susceptible individuals can sustain a new cycle of rabies infections acquired upon contact with other infected colonies thus repeating the rabies circulation cycle [31].

In cattle symptoms of rabies start about one month after the animal was bitten by a rabid vampire. Cattle usually die in clusters, presenting as a livestock rabies outbreak. Bats that used to feed on the deceased cattle will look for other food sources, and then find humans. Vampire bats need to feed at least once every 24–48 h otherwise they will become dehydrated and die [32, 33]. The report of a cattle outbreak serves as an alert for heightened human risks, because bat bites to humans will likely increase after cattle blood availability is reduced. Bats incubating rabies or recovered from rabies can find accessible humans living in the area as new regular prey. When an accessible human population lives in proximity to a cattle outbreak, there is an approximate lag of a month after the outbreak in cattle till the first human rabies case. This, in turn, provides enough time to intervene by administering rabies prophylaxis to prevent human cases. The standard response for such a case was outreach to the affected locations and survey of human bite cases; rabies post-exposure prophylaxis (PEP) was then administered only to bitten individuals. In nearly all cases only rabies vaccine was given, omitting rabies immunoglobulin, though it is part of the PEP in most national rabies regulations. Sometimes, the colony causing a cattle outbreak has no other animal or accessible human food sources available for its size, so that the bats are forced to look for neighboring food sources in their flight range, which is up to ~16 km [34]. Another village without cattle can be targeted where bats end up feeding on people. Due to the remote location and dispersion of the villages in the Amazon Basin in Peru, reporting the increase of bites to humans is never timely, although it is mandated by the national surveillance system. A similar situation occurs with the detection and report of rabies cattle outbreaks. They are reported late, most times no samples for testing are obtained, resulting in lack of the required laboratory confirmation for rabies virus. The time lapse between detection, reporting, and mobilization to remote places delays the outbreak response, resulting in human deaths. The usual casualties in those human rabies outbreaks are mostly indigenous minority ethnic groups, making rabies a deadly consequence of persistent disparities in healthcare of the vulnerable

native populations of the Amazon Basin. Clusters of >10 human deaths are not unusual. Further consideration to this issue is the fact that, as a rule, the index case is a child and almost all deaths occur in children from native people [35]. Due to the body size of children, neurotropic viruses, such as rabies virus, have shorter distances to reach the CNS and the onset of rabies encephalitis is faster than in adults. To make things worse, bites to the head and nose are most common in children. Historically, the health system responded to a human rabies outbreak only after at least one death in a child had been reported. Consequently, it is most likely that the interventions through postexposure prophylaxis only prevented adult deaths if any at all.

Finally, the main vector control activities aim to destroy vampire bat colonies, using methods that are unspecific for vampire bats and usually affect other bat species as well. A common intervention is the capture of a few vampire bats, then apply an ointment with an anticoagulant, such as warfarin, in the bats' back and release them. The vampire bats return to the colony and will spread the ointment to other bats when grooming, a behavior of *D. rotundus* that guarantees close physical contact among individuals in the vampire bats' refuge [36–38]. The use of anticoagulant ointments for almost 50 years in Latin America and in Peru did not stop cattle rabies outbreaks [39].

Typically, most human rabies outbreaks due to vampire bat bites follow that pattern, such as in the 2015 outbreak in Soledad, Loreto, Peru, at the Basin of Rio Curaray. This outbreak, which was suspected after a child presented to a hospital with symptoms of rabies elicited a fast and aggressive response, which still could not avoid rabies infection and death in three additional children. A significant number of cattle death that occurred one month before went unreported and was only documented after the human outbreak alert was issued [40]. The timeline of that outbreak is shown in Fig. 1 following the sequence explained above.

Because all traditional interventions for rabies control and standard prophylaxis recommendations failed to stop human rabies outbreaks, and there are no effective ways to interfere with rabies in the vampire bats, human population became the only accessible target for intervention. When rabies virus exposures to humans are endemic and occur frequently, it is justified to consider strategies for massive immunization of the human population residing in endemic places. Introducing such a strategy requires the update of the national regulations for rabies control and changes in the corresponding public health policy that makes massive rabies immunization mandatory in high-risk areas for vampire bat rabies.

Basis for a Massive Rabies Pre-exposure Prophylaxis (PreP) Campaign in Peru

From 1975 to 2010, a total of 292 human rabies deaths associated with vampire bat bites were reported in Peru, 45% [131] of the cases were from only one province, Condorcanqui Province in the Amazonas Department [41]. All of the deaths were reported in children at or below the age of 15 years. Condorcanqui covers 39,241

km^2, and 93% of its population belongs to the Awajun, an ethnic group native to Peru and the Ecuador Amazon Basin. With a population of only 50,000 that single province was the source of more than 80% of all rabies deaths from all of the Americas before a massive vaccination program was developed [28]. Three districts compose the Province, Nieva, Rio Santiago, and Cenepa, all of them were affected by vampire bites and human rabies outbreaks. These persistent rabies foci were addressed for years after each outbreak report by rabies PEP using suckling mouse brain rabies vaccine (SMBV), administered only to people considered exposed, according to national rabies regulations [42]. The criteria to initiate PEP was self-report of a bat bite noticed within the recent 6 months, which resulted in vaccination of approximately 20% of humans residing in the affected villages (SR, unpublished data). Laboratory confirmation of human rabies outbreaks due to vampire bat bites was a major challenge. Lateness of interventions and refusal of the patients' relatives usually prevented autopsies or collections of post-mortem samples. In only 2% of the suspected cases rabies were confirmed by the National Rabies Reference Laboratory, while 98% of cases were only diagnosed based on symptoms [42, 43].

An increase in the frequency of human rabies outbreaks and the total number of deaths per outbreak was observed in several places of the country, especially in the Condorcanqui Province since the year 2007 [44]. The situation triggered early considerations for a different intervention approach, including massive vaccination, as technical documents by WHO started to include recommendations for PreP in high-risk areas [12]. An obstacle at that time, was the use of a nervous tissue vaccine (NTV), the suckling mouse brain vaccine (SMBV) also known as Fuenzalida-Palacios vaccine in Peru, which was manufactured locally by the Peruvian National Center of Biologics Production. NTVs are not recommended by the WHO due to their association with severe neurological adverse reactions [4, 45, 46]. Because of that, the use of SMBV was inappropriate for mass vaccination campaigns. In Peru, rabies prophylaxis is given free to the public under a national Ministry of Health program. Peru slowly switched to the use of rabies cultured cell vaccines (CCVs) starting in 2009, and by 2014 finally banned the use of NTVs and stopped production of SMBV [47].

In 2011, the district of Imaza, the capital of the Bagua Province, reported its first human rabies outbreak, with 22 deaths. Imaza is highly populated, and adjacent to the Condorcanqui Province to the East and to a major city, Bagua, to the West. The Imaza outbreak triggered an intense emergency response and the decision to proceed with a massive PreP campaign using CCVs [41, 48, 49].

Additionally, the government office for Citizens Rights Protection, Defensoría del Pueblo, issued in 2015 a report on the human rights of the Amazonic Native Peoples of Peru, and examined the situation of populations exposed to rabies, calling for the implementation of effective outreach policies for their remote Amazon Basin populations, to stop human rabies outbreaks among indigenous communities [50].

In Fig. 2, the human rabies cases due to vampire bat bites reported in Amazonas Department, including Condorcanqui and Imaza, are compared with cases from the rest of the endemic areas in Peru. The intervention with massive PreP started in 2011

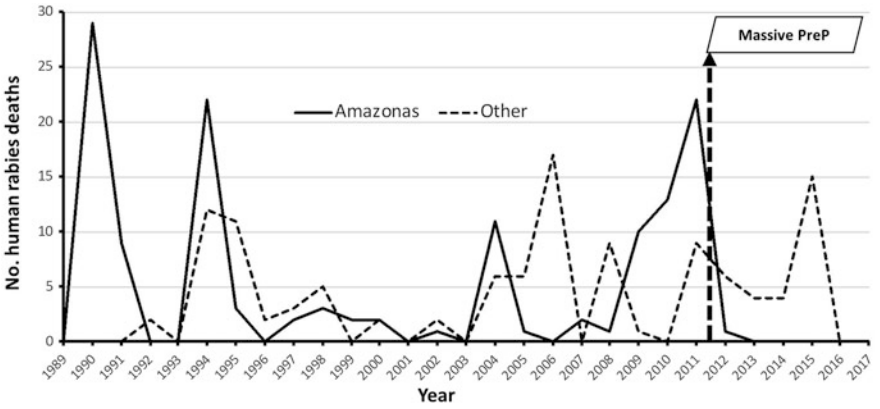

Fig. 2 Human rabies cases due to vampire bat bites in Peru, 1989–2017. Cases reported by the Amazonas department were separated to compare them to other endemic areas in the country. Data source: Direccion Regional de Salud Amazonas, and Peru Ministry of Health

Table 1 Reports of animal bites by region endemic to vampire bat rabies, Peru 2009–2013

Region	2009	2010	2011	2012	2013	Total	%
Amazonas	1576	5714	2145	1733	833	12,001	59.2
Cusco	50	169	36	441	20	716	3.5
Loreto	1122	856	1458	1380	590	5406	26.7
Junin	119	415	179	142	29	884	4.4
Others	465	224	295	229	41	1254	6.2
All	3332	7378	4113	3925	1513	20,261	100

Table translated from Estrategia Nacional de Zoonosis-MINSA. Informe 37-2015

in Amazonas preventing cases there, while other endemic areas continued presenting human rabies outbreaks.

The data from animal bites in the endemic area of Peru in Table 1 indicate the two most important places for exposures, Amazonas and Loreto. At the beginning of the PreP campaign, Amazonas had most human rabies cases while Loreto at that time of the decision to combat vampire bat rabies with massive PreP, did not report recent outbreaks. Data from animal bite surveillance were used to include areas other than Amazonas in the national Massive PreP program [43].

Assessing the Risk of VBR for Humans

The presence of vampire bats' colonies is documented in all regions of Peru, but rabies circulation in the sylvatic cycle in vampire bats is only documented in the Amazon Basin ecoregion [44, 51, 52]. Rabies in cattle in that part of the country is common and caused by vampire bat transmission. While vampire bats' rabies is considered a risk for the entire Peruvian Amazon Basin territory because of the

Fig. 3 Typical homes in remote villages in the Peruvian Amazon Basin. Often built on an elevated platform along or close to the riversides. Datem del Marañon, 2010, Loreto, Peru. Picture credit: Sergio E. Recuenco

presence of vampire bats and rabies virus circulating among bats, not every location where rabies was reported in cattle or other domestic animals presented human cases [51, 53]. Circulation of rabies virus among vampire bats is not sufficient to trigger human rabies outbreaks in local populations. Bites of rabid vampire bats to people are required. Living conditions make a difference, regarding access of vampire bats to human individuals at night when they are sleeping. Vampire bats bite people who are soundly asleep and they can feed leaking blood from the wound for several minutes, providing repeated contact with bat saliva. Due to cultural reasons and tropical climate, traditional rural houses of native populations in the Amazon are mainly made with local materials from the forest, lacking doors, or closed windows, with many openings in walls and floors allowing for entry of flying bats at night. Homes are usually elevated on wooden platforms because villages are located in seasonally flooded land. The platforms also have openings granting bats access to dwellings. Living in a typical Amazonian home as shown in Fig. 3 is an important risk factor for human rabies outbreaks [23, 54]. Home modification had been proposed as an alternative intervention decades ago, but no changes were ever implemented [55]. Housing styles of rural Amazonian populations remain unmodified until now.

Although vampire bat bites are common events across all humans residing in the Amazon Basin, the risk for human rabies in a given location is only acknowledged after reports of human cases [35]. A 2010 study, in Datem del Marañon, a province adjacent to Condorcanqui, provided further undeniable evidence that Amazonian populations with no report of previous human rabies cases or outbreaks in cattle were exposed to rabies virus and at risk for symptomatic infections. Besides finding the highest ever reported frequencies of vampire bat bites in people, the study also detected rabies antibodies in 11% of the surveyed people; none of them had ever been vaccinated against rabies, nor had they not presented neurological symptoms in the past. Antibody titers in these individuals were low and it is assumed that they were induced by repeated contacts of the individual with very small amounts of

rabies virus. While such findings triggered a new hypothesis regarding human rabies immunity, it became the strongest evidence for urgent intervention in all remote Amazon native communities to prevent future human rabies outbreaks [24].

Local populations are mostly unaware that vampire bat bites transmit rabies, and usually do not realize the association of a bat bite with the onset of rabies symptoms weeks later thereby delaying the recognition of risks and reporting to health services [24, 56, 57]. Mosquito nets are used in some areas to prevent vampire bat bites, but those nets are usually in bad shape, they are not renewed for years, and misused allowing bat bites to individuals under the net, or even several children under the same net.

In general, vampire bat bites are daily life events in rural Amazonian populations and remain underreported and untreated. The rabies risk for human populations living in the conditions explained above were considered continual for the purpose of planning a massive anti-rabies immunization campaign as a radical intervention to stop human cases.

The Massive Rabies PreP Campaign Plan

The recognition of Condorcanqui Province as a persistent human rabies focus, and its adjacent location, Imaza, with decades of failed control strategies, justified a radical change from qualifying for rabies prophylaxis only after self-reported exposures to immunizing all residents in a given high-risk area. High frequency of bat bites, evidence of rabies circulation, vulnerable housing conditions, lack of protective measures among the population, lack of tools for vector control, and remote location of the affected villages that make timely responses difficult, were the factors supporting the need for massive immunization given the high likelihood for any individual in the population to be exposed to bites from rabid bats [23, 24, 35, 41, 51, 58].

The massive Rabies PreP campaign was approved in agreement between the Ministry of Health and the Government of the Amazonas Region in July 2011 [41]. The plan had the goal to prevent new human rabies outbreaks in the targeted areas and included the following objectives:

1. "To schedule the order of places to intervene, identification and prioritization of targeted classifying the epidemiological risk under several criteria.

   (a) Area endemic for vampire bat rabies
   (b) No human rabies cases in recent 6 months
   (c) No rabies cases in cattle within last 6 months
   (d) Unchanged frequency of vampires bat bites in places with animal bite surveillance.
   (e) Location with no history of intervention with rabies vaccination (PEP in outbreak response)

(f) For villages with history of PEP intervention, priority was given to places with: intervention >1 year in the past, low percentage of population that received PEP, proximity to location with recent outbreaks or documented rabies circulation (>20 km distance).
(g) Important and recent ecological changes

2. To achieve immunization of 100% of the population of each village and location that was selected according with the criteria above.
3. To strength the capabilities of the Regional Laboratory for Rabies Diagnostics.
4. To achieve a rabies surveillance system strong and sustainable for high rabies risk areas
5. To obtain community support and participation of the population in the selected locations, and improvement knowledge and practices to prevent rabies
6. To inform targeted population about the campaign and preventive measures for sylvatic rabies
7. To properly train and engage the required human resources and to efficiently apply their knowledge
8. To contribute with evidence to allow the evaluation of the results and impact of the rabies prophylaxis intervention in vampire bat risk areas
9. To evaluate process and results of the applied rabies prophylaxis strategies in areas with rabies risk transmission and bat bites." [41]

The multi-year plan was executed with collaborations between the Regional Governments of the involved regions, the Ministry of Health, and the Peruvian National Institute of Health. The cost of the plan was estimated to be approximately 4,111,000 US dollars with an estimated cost of the vaccine of 3,560,000 US dollars. The vaccine cost includes the estimated commercial value of the HDCV used and received by donation to the Ministry of Health, and the PVCV the government bought to complete the needs of the plan. The plan considered an estimated cost per immunized person of 69 US dollars [41]. If we consider that every year ~20 human deaths were reported only in Condorcanqui Province, and only one massive intervention was needed to stop rabies deaths, an early account for cost per life saved, indicate an investment of 205,550 dollars per life saved in the first year, but after five years it is reduced to 41,000 dollars, a cost that will be reduced every year without new cases [59].

With available human rabies vaccine, the Peruvian Ministry of Health approved an expedited start of the Massive PreP Program plan. Additional amounts of vaccine were required to complete the program timely. This started a parallel process for provision without interruption of the vaccination schedules. The PreP scheduled used a 3-dose (days 0, 7, and 28) IM regimen given into the deltoid region, and the external side of the thigh for children ≤2 years old. This PreP schedule was recommended by WHO at that time [42, 60].

Two hundred and four localities were selected as priorities, targeting 40,904 people, including 18,635 children ≤15 years old. A larger number of localities were included during the campaign as soon as more information was gathered and

Table 2 Population immunized with rabies PreEP and coverages by risk region, 2011–2014

Region	Target	2011	2012	2013	2014	All	% of target
Amazonas	82,553	15,368	19,277	20,723	16,019	71,387	86.5
Junin	1800	0	2580	2505	0	5085	282.5
Cusco	15,046	0	0	9645	0	9645	64.1
Loreto	21,886	0	0	4760	0	4760	21.8
All regions	121,285	15,368	21,857	37,633	16,019	90,877	74.93

Table translated from: Estrategia Nacional de Zoonosis-MINSA. Informe 37-2015. Resultados plan de vacunación antirrábica de pre-exposición en comunidades en riesgo de rabia de la Region Amazonas—Perú 2011–2014

the plan was updated. During 2011, the number of intervened localities was 268, of those 86% were from the Condorcanqui Province, and 14% from Bagua Province, both from Amazonas, immunizing 13,986 people. The scheduled was initiated in 15,242 people, and nobody was lost to follow-up in Condorcanqui, while 2.4% were lost to follow-up in Bagua. During that year, 46% of the population in Condorcanqui was immunized. The absence of rabies cases in children in the intervened areas in 2012, is a strong and tangible evidence for the impact of the intervention. There were only two cases reported, both in adult individuals who formally refused the immunization [41, 43].

Table 2 presents the progressive incorporation of endemic areas that were included in the vaccination campaign. The new plan prioritized 423 communities in Condorcanqui and Bagua, 91% of them were native communities, and all were located surrounding the areas of higher risk for sylvatic rabies. By 2014, 71,387 people from Bagua and Condorcanqui completed the schedule, achieving 86% of coverage, and no serious adverse event were reported from the population immunized [43].

In 2012, a new human rabies outbreak in Cusco Region resulted in the evaluation of the affected area with the plan criteria and its inclusion in the Prep campaign plan. Qualifying risk areas in the Junin Region and Loreto Region were also included [43].

The most recent update of the plan, in 2016, maintained same PreP schedules in concordance with WHO recommendations [11]. The geographic area for intervention was expanded to include provinces in the Departments of Amazonas, Ayacucho, Madre de Dios, Ucayali, Cusco, Junin, Pasco y Loreto, as presented in Fig. 4 [61]. Other regions at risk, but related to migratory and floating populations, such as Huanuco, Puno, Apurimac, Pasco, and San Martin, are under evaluation for future inclusion in the PreP Program.

Risk Assessment for a Massive PreP Intervention

While massive rabies PreP interventions halted human rabies cases where applied, it is a costly and logistically complicated operation and consideration for a candidate region requires examination of several key risk factors. The factors listed in Table 3 are specific for vampire bat rabies in the Peruvian Amazon Basin, and can be similar

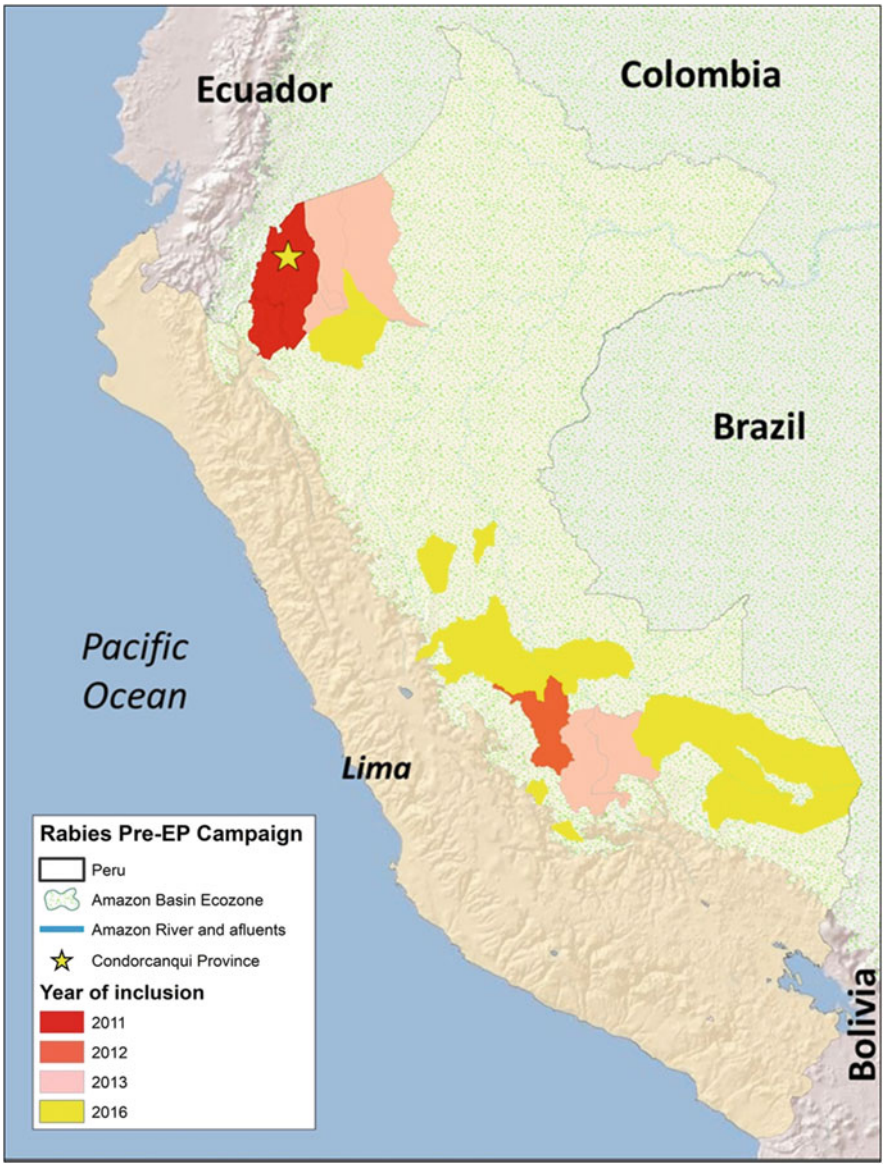

Fig. 4 Provinces included at National Massive Rabies PreP Program by year 2011–2015. Based on data from Ministerio de Salud-MINSA Peru

for other places in the region. Migration and tourism are included due to continuous development of different activities attracting travel to endemic areas resulting in precarious new settlements and worker camps, likely accessible to vampire bats. Places attractive for tourists, for adventure, and ecology exploration, are likely

Table 3 Risk factors for vampire bat rabies for planning massive PreP intervention in endemic areas

Risk factor	Situation in Peru	Evidence source	Value	Considerations
Exposure frequency	High frequency of vampire bat bites in rural populations of the Amazon basin	Animal bite surveillance	Very high	Associated to homes lacking walls, or closing doors and windows
Exposure care	Usually neglected in rural villages	KAP surveys	High	Bites considered ordinary event due to high frequency
Health care/ PEP access	Small rural villages are remote without accessible healthcare services	Geographical distance, length of travel	High	Most large urban centers have accessible services
Rabies virus circulation	All Amazon basin is endemic	Cattle rabies outbreaks, rabies surveillance	Very high	Lack of report and rabies laboratory surveillance in most of the endemic region
Protective measures against exposures	None effective measure in use, mosquito nets used mostly incorrectly	KAP survey	Medium	In a typical Amazonian home, bats have not barriers to access humans
Tools for vector control	Vampiricide used during outbreak response, late to prevent any human death	Rabies surveillance	Medium	Rabies persist despite more than 50 years of vampiricide ointment use
Human mobilization	High frequency or migration in the Amazon basin due to extractive resource industry activity	Signs of economic legal/ illegal activity (deforestation, mining, other)	Medium	Increased number or susceptible population hard to monitor
Cultural approach	Rabies signs sometimes attributed to magical reasons	KAP survey	Medium	In places with historical high frequency of bites
Previous human rabies outbreaks	Repeated human outbreaks in same districts through ~40 years, but new locations keep adding	Rabies surveillance	High if present	Lack of human population, or sufficient animal food sources available can result in no cases reported
Previous history animal outbreaks	Widespread cattle rabies across Amazon basin	Rabies surveillance	High	Lack of reporting in low density cattle farming
Tourist destination	Increase of ecotourism in Amazon Reserve Parks	National economic information	Low	Normally tourist areas not endemic, and PEP accessible in case of exposure

accessible to health care services and are of less concern but can result in increases of floating populations to areas with high risk for rabies and medium to little health care access. Assessing the risk can assist to prioritize places, and improve efficiency of available resources allocation to immunize the population at rabies risk.

Massive Immunization and PreP Schedules

The massive administration of a vaccine implies preparedness for potential adverse reactions and the adequate and timely provision of the needed vaccine doses to achieve targeted coverage [4]. With the use of CCVs, adverse reactions are minimal in frequency and severity, with only local mild events. The Peruvian PreP Program plan included monitoring vaccine adverse events [61]. After more than 90,000 doses administered no serious adverse events were detected [43]. The major challenge regarding administering the vaccine was accessing the remote locations and completing the follow-up for the three-dose schedule recommended by WHO at the time the first campaign. To maximize public health impact and cost benefit in remote locations, rabies PreP can be given with other vaccines to children and adults. In that regard, all current WHO prequalified human rabies vaccines have not contraindication for simultaneous administration with other vaccines [4, 62].

One important consideration, specific for high-risk exposure areas, is an assessment if an individual scheduled to receive PreP has recently been exposed to rabies by a vampire bat bite and should receive PEP, which requires additional vaccine doses and rabies immunoglobulin instead. In absence of an active rabies outbreak in cattle or humans, it is acceptable to assume the viral circulation is low or unlikely at that moment and administer PreP to all individuals disregarding recent vampire bat bites.

The presumed protective level of rabies neutralizing antibodies, 0.5 IU/ml by WHO standards, is reached in most individuals 7 days after the first PreP dose, and in ~100% after the second dose [63, 64]. PreP induces a sustained antibody response that may decay after several years. At that time a single boost will elicit a potent anamnestic response [65, 66].

WHO recently changed recommendations for rabies PreP. It now recommends two rather than three doses of vaccine given IM (1 ml or 0.5 ml depending of vaccine presentation) on days 0 and 7 into one site or, or ID (0.1 ml) given on days 0 and 7 into two sites. The third dose is no longer viewed as necessary and there is no need for further boosts for healthy individuals who are not exposed. A third dose can be administered on day 28 to immunocompromised individuals [4]. This update likely will reduce the cost of the rabies vaccine and simplify logistics for implementing mass vaccination campaigns in remote villages.

In 2018, WHO issued an official position by recommending the use of PreP for populations living in areas where vampire bats' bites are present [62]. Additional areas of the South American continent that were considering massive PreP, will likely implement such programs after carefully assessing vaccine availability and program costs [23, 66]. Using the now recommended ID PreP schedule will save a

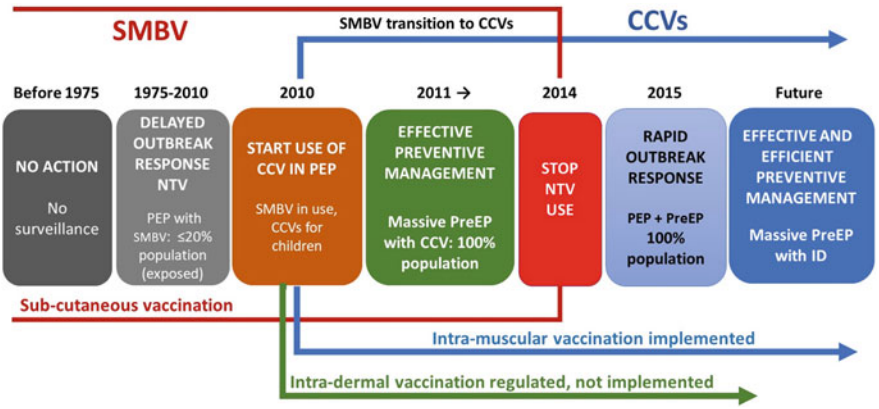

Fig. 5 Evolution of the response to vampire bat rabies in humans. SMBV Suckling mouse brain vaccine, CCV Cell cultured vaccine, NTV Nervous tissue vaccine, PEP Postexposure prophylaxis, PreP Pre-exposure prophylaxis, ID Intradermal

substantial amount of vaccine and minimize waste of vaccine leftovers in 1 ml or 0.5 ml vials when using 0.1 ml ID doses, because large numbers of people will be vaccinated at the same time [63]. Planning the most efficient way of vaccine distribution will be key to shorten the time to cover all humans at risk in the Amazon Basin across the continent and using the WHO recommended ID schedules will be key to reducing the cost. Although ID PreP schedules are mentioned in most national recommendations for rabies control, it is rarely used in most Latin American countries [47]. Figure 5 shows the evolution in the response to human rabies outbreaks caused by vampire bat bites, from no response in early years to the current massive PreP interventions. The obvious next development for this evolution is to switch to ID vaccination in rabies prophylaxis.

Recent research is examining shorter schedules for PreP, notably one-day schedule with 4-site 0.1 ml ID doses [67]. Shortened schedules would be ideal for remote areas with difficult access and places with nomadic native populations. Research into shorter vaccination schedules in the Amazon Basin populations can provide insight for logistic improvements leading to more efficient and faster massive PreP interventions.

Conclusion

Although the idea of massive PreP immunization was explored decades before for canine and bat rabies, the first massive intervention as an official public health policy and funded by a government, did not occur until 2011 in Peru, and is expected to expand as public health policy to other Latin American countries endemic for vampire bat rabies.

While in canine rabies, the vector is mostly accessible for intervention through immunization, and population control, for rabies transmitted by vampire bat bites, there is neither efficient nor efficacious method for animal vector control available. The search for new technologies that would allow rabies control among bats is neglected.

In the present, the lack of strategies to control rabies in bats, justifies inventions with rabies PreP targeting human populations at risk for vampire bat bites. The Peruvian PreP plan was successful to drastically reduce human deaths due to rabies by changing the rabies-specific immune status of the population without changing the frequency of bat bites to the population. Further research and strategies are needed to diminish the incidence of bat bites and reduce the rabies risk to the population in a more sustainable manner. In the meantime, massive PreP administration to very high-risk areas is feasible, safe, and an urgent strategy to stop the human deaths due to rabies in the Amazon Basin.

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Rabies Prevention in Asia: Institutionalizing Implementation Capacities

Mary Elizabeth G. Miranda and Noel Lee J. Miranda

Abstract

Rabies in Asia and Africa contributes to over 99% of human rabies deaths that occur in the world today. The vast majority or 60% of these deaths are in Asia. Practically, more than four billion people in Asia or about 60% of the world's population are at risk of getting rabies where an estimated 96% of documented human cases are from an infected dog bite. Canine-mediated rabies is one of the few communicable diseases that can possibly be eliminated by currently available vaccines and tools for veterinary and public health interventions. With a more comprehensive and integrated approach, it is expected that dog rabies will be eliminated in target areas, and there will be an eventual decline and disappearance of human rabies cases. The burden of rabies is primarily on human health but the disease control has to be focused on the animal source. The ultimate goal of a truly regional disease program is to control and eliminate dog-mediated rabies and protect and maintain rabies-free areas in Asia. Current regional efforts aim to strengthen the intercountry coordination, and technical and institutional capacities to manage dog rabies elimination programs. The regional and national implementation efforts provide strategic direction and cooperation to ensure successful implementation of rabies control measures and eventual elimination. The focus areas include human rabies prevention through pre- and postexposure prophylaxis, mass dog vaccination, surveillance and epidemiology, laboratory diagnostic capability, public awareness and risk communication, legislation, dog population management, and establishment and protection of rabies-free zones/

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H. C. J. Ertl (ed.), *Rabies and Rabies Vaccines*,
https://doi.org/10.1007/978-3-030-21084-7_6

areas. Existing mechanisms for implementation, when applied, give emphasis on One Health collaborations.

Situation in Asia and Regional Initiatives

For most countries in Asia, canine rabies is endemic and the majority of human rabies exposure results from dog bites particularly among children. The estimated number of human deaths across Asia and Africa is approximately 59,000, with over 3.7 million disability-adjusted life years (DALYs) and 8.6 billion USD economic losses annually [1]. The vast majority of these deaths is in Asia (59.6%). India, with 35% of human rabies deaths, accounted for more deaths than any other country. Rabies is often neglected when health and agriculture (animal health) agenda and budgets are set even if the costs and economic benefits have long been described [2, 3]. It continues to be neglected and very often its public health impact is minimized by other priority infectious diseases like dengue, malaria, tuberculosis, and HIV. Reliable data indicating the actual incidence of human rabies and rabies risk exposures are often lacking or non-existent in many countries, leading to the global number of human deaths that is significantly underreported [3–5]. Canine rabies is not only a major burden in endemic countries where thousands of human deaths occur annually, but also in previously rabies-free areas where risks of re-emergence have been increasing over the last decade [6–10].

The burden of canine rabies is substantial, even though the disease is entirely preventable. Dealing effectively with the problem is contingent on investing in the control at the animal source, which has long been lacking. Long-term mass dog vaccination with high enough coverage could reduce health sector and societal costs with more rational and judicious use of postexposure vaccination [11, 12]. Disease elimination is feasible with currently available vaccines and disease control methods; however, innovative financing models are required to overcome institutional barriers.

In 2001, the First WHO Interregional Consultation on Strategies for the Control and Elimination of Rabies in Asia laid down the impetus for many Asian countries to promote and pursue the elimination of canine rabies to eventually eliminate the disease in human populations [13]. Asian countries were urged to develop comprehensive national plans with improved access to modern human vaccines and application of new economical postexposure treatments, better disease diagnosis and surveillance, and processing of data at the national, regional, and global levels, intersectoral collaborative efforts for dog rabies control and plans to expand public and health care worker awareness regarding rabies control and prevention.

The 2008 Association of Southeast Asian Nations ASEAN Call for Action toward the elimination of rabies in the ASEAN Member states and the plus three countries (China, Japan, and Korea) by 2020 demonstrated the key importance attached to rabies control at a political level [14]. The ASEAN Rabies Elimination

Strategy (ARES) was developed in 2015 to provide a strategic framework for the reduction and ultimate eradication of canine rabies in ASEAN Member States. The strategy describes an integrated One Health approach that brings together the necessary sociocultural, technical, organizational, and political pillars to address this challenge. The ARES was designed to complement the existing subregional frameworks developed to control and eliminate human rabies, such as those developed by the ASEAN Expert Group on Communicable Diseases (AEGCD) in 2010. In South Asia, considering the importance of consolidating achievements in rabies control in Member countries, the WHO Regional Office for Southeast Asia has developed a regional strategy for elimination of human rabies transmitted by dogs (2012) [15].

In the Middle East and Central Asia, human cases still occur, and dogs are the main vector. These regions plus countries of North Africa and Europe, belong to the Middle East and Eastern Europe Rabies Expert Bureau (MEEREB), an interregional rabies prevention and control network. In 2015, MEEREB has called for elimination of dog-transmitted rabies through vaccine and rabies immunoglobulin stockpiles and implementation of a One Health approach to achieve rabies eradication [16].

Animal Rabies Prevention

Across the continent, there is a marked increase in community-based initiatives for domestic animal vaccination and control with increased government support to funding and better program implementation. Animal rabies control activities vary across the region [17–20]. Many national and subnational programs and demonstration projects have proven that proactive mass dog vaccination is much more effective at controlling rabies and less costly than campaigns that vaccinate in response to the occurrence of cases. Control through proactive vaccination followed by two years of continuous monitoring and vaccination should be sufficient to guarantee elimination from any area not subject to repeat introductions [6]. The degree of success of national and global canine rabies elimination efforts, however, depend heavily on effective epidemiological surveillance, which should ensure that intervention impacts can be monitored through time and outbreak responses initiated where necessary. It is recommended that rabies control programs ought to be able to maintain surveillance levels that detect at least 5% (and ideally 10%) of all cases to improve their prospects of eliminating rabies, and this can be achieved through greater intersectoral collaboration [6].

Rabies is a community-based problem that requires a well-organized and funded community-based approach. Many countries need to strengthen their community-based programs and implementation platforms, especially where government lacks the capacity and effective governance to mobilize community efforts [18, 19]. In these settings, well-organized community efforts that aim to support or augment existing government rabies elimination programs are much desired. In countries such as Indonesia, Philippines, Sri Lanka, and Thailand, these are often facilitated by

nongovernment organizations and civil society organizations closely coordinating with the local government and private sector groups.

There are practical bottom-up approaches that can be appreciated by governments. Effective community-based approach seeks to strengthen the capacity of families, individuals, organizations, institutions, and systems to support disease programs and outbreak responses. It is expected to contribute in programming to reduce rabies risk and address community vulnerabilities, and enhance community and institutional resilience, being sensitive to the issues directly confronting communities, and desiring to support families and all sectors involved (whole-of-society) to take the necessary actions to reduce dog bites and rabies transmission risks. Working through the lowest unit of a community, the family or household unit, is central, as "more resilient families are the foundation of more resilient communities." Individual family units should be fully aware of rabies threats and the required interventions and be the first to take action when these threats appear, such as reporting dog bite incidents to community leaders. The ability of a nation to eliminate rabies starts at household levels—with family members understanding the risks of rabies and being able to systematically mitigate spread, and therefore ensure neighborhood rabies security. Fostering private sector commitment to building and empowering communities through their corporate and human resource is another key element to effective community efforts.

An integrated approach is the most effective way of protecting humans from canine rabies, as the infection is maintained in domestic dog populations [11]. A number of countries have achieved considerable success in canine rabies elimination through mass dog vaccination. The feasibility and cost effectiveness of this approach have been strongly advocated in recent years, with major international public and animal health organizations declaring global canine rabies elimination as a realistic goal.

Human Rabies Prevention

Postexposure Prophylaxis

Significant progress in the production of rabies vaccines for human use that are low cost, rapidly immunogenic, safe and practical to use has led to increasing accessibility to timely and appropriate PEP. In the early 1990s, Thailand pioneered the PEP intradermal (ID) regimens using cell culture-derived vaccines [21]. From the time it was endorsed by WHO in 1992, accessibility further improved due to increase in numbers of animal bite management centers, and better quality of services [21, 22]. This eventually eased out the production of mammalian nerve tissue-derived vaccines. In the Philippines and Sri Lanka, since mid-1990s, animal bite treatment centers were established in government hospitals and major health facilities [21, 22]. Minimum essentials include training for MDs and nurses in the proper management of patients and rabies exposures, cold chain, and systematic

recordkeeping or registry. To date, ID regimens are the first line in majority of the national rabies prophylaxis protocols and recommendations.

Rabies immunoglobulin of equine origin (ERIG) are in short supply throughout the world and particularly in Asia, the demand is high [19]. ERIG available in Asia is either manufactured in Europe, India, or China. Though ERIG is considerably cheaper than human origin immune globulin, modern production of immune sera generates highly purified and safer products of better quality. Producers of ERIG should be encouraged to continuously aim for consistent antibody levels and the least incidence of adverse reactions among patients.

Pre-exposure Prophylaxis

Current recommendations for pre-exposure schedule use ID injections of cell culture vaccines as a cost-reducing alternative for developing countries. As a strategy to augment human rabies prevention measures, childhood rabies immunization has been included in the national programs of countries like the Philippines and Vietnam.

Human Rabies Vaccine Production in Asia

Human vaccine production capacities of Asian countries have improved greatly. Rabies vaccine supplies come from a mix of private and public manufacturers in several Asian counties and are mainly for domestic use, but some manufacturers have the potential to export vaccines. Over the last decade, both private and public vaccine manufacturers in Asia have exerted extra efforts to meet stricter government registration requirements as countries adhere to international and regional GMP (Good Manufacturing Practice) standards and vie for WHO pre-qualification. The National Regulatory Authority (NRA) in most countries generally enforces their local GMP standards, which tend to be stricter and more demanding to the producers [23]. The procedure for approval of newly introduced vaccines is very much in place, involving the conduct of complete preclinical and clinical testing and establishing lot consistency prior to approval for marketing. Most countries have functional NRAs/National Control Laboratories (NCLs), which provide overall control on the vaccine production process and final product quality. Exceptionally, a few countries still lack the capacity to perform rigid laboratory testing.

Demand and Supply Human rabies vaccine manufacturers are growing in number most markedly in China and India with more than 15 manufacturers serving a combined population of more than 2 billion with an estimated demand for rabies vaccines of about 30 million doses or about 6 million full-course treatments per year. The vaccine production levels typically range from 100,000 doses to 10 million doses per year (for cell-based production facilities), with some producers in China and India upgrading their capacities to produce more [23].

Vaccine Types China and India discontinued the production of mammalian nerve tissue origin (NTO) vaccines in 2001, and Vietnam stopped producing the suckling-mouse brain vaccine, which has been in use over 30 years, in 2005 [23]. It now imports cell line-based vaccines, and they have also modernized their main vaccine manufacturing plant (GMP compliant) located in Hanoi. All these manufacturers follow the WHO standard requirements, as well as refer to USP requirements. There are essentially 5 types of modern vaccines, according to the substrate, that are produced mainly in China and India, namely the Primary Hamster Kidney (PHK) cell, Vero cell, Human diploid cell (MRC-5), Embryonated duck egg, and Chick embryo cell. Of these, the Vero cell is the only continuous (animal) cell line. All these vaccine types undergo concentration and purification processes by either zonal centrifugation or tangential filtration and gel chromatography. The virus strains being used include the PV (Pasteur Institute Paris or CDC) and PM (Wistar Institute), and in China they also use their locally derived strains. Vaccine preparations are either liquid or freeze-dried single dose (0.5 or 1.0 ml) in glass vials and are administered according to the Essen regimen [23].

Quality Asian vaccine manufacturers generally apply in-process control measures that include sterility tests, ELISA, SRD, and NIH potency testing. National Regulatory Authorities only issue marketing licenses if complete testing of vaccines, including preclinical and clinical studies, has been conducted. Laboratory testing regimens applied essentially follow WHO requirements, and Lot Release systems are being constantly reviewed and modified. In China, a system of random testing of production lots and post-market surveillance/testing and product recall are being strictly put in place. Random lot testing generally includes tests for sterility, safety, and potency (NIH method). Most rabies vaccine manufacturers (public and private) seek to be WHO pre-qualified as they consider it advantageous to the marketing and worldwide distribution of their products [23].

Costs In China and India, the cost of modern locally produced vaccines range from 3 to 7 USD per dose. In China, imported vaccines are 10–14 USD per dose. In Southeast Asia, imported vaccines typically range from 3 to 10 USD per dose.

Hold Backs and the Way Forward Some countries that intend to start their local modern rabies vaccine production need support to establish cell line-based vaccine production, such as seed virus, cells, technology transfer, and funding for equipment or facilities. As WHO prequalification is sought for rabies vaccines, manufacturers observe that the process takes too long. As more new manufacturers join in, the demand for training of personnel on GMP must be addressed by all stakeholders. The reliability of the currently prescribed NIH potency test is a major problem that manufacturers face in the production and control of rabies vaccines. Essentially the NIH test gives varying results depending on the laboratory and the status of the mice which the test utilizes. Some manufacturers have also questioned the need to conduct the stability testing of vaccines on a per batch basis [23].

In general, countries are able to follow the WHO requirements for human rabies vaccine production. The complete replacement of NTO vaccine with cell line-based rabies vaccines has been accelerated, as India and China have demonstrated the feasibility of domestic commercial vaccine production. Countries generally have the desire to produce better quality vaccines but are concerned about the effect on supply, and how to get the production of cell line-based vaccines started. Regional supplies of relatively inexpensive vaccines will surely influence the decisions of countries to produce their own cell line-based vaccines. It would be advantageous to countries if WHO introduced a system of recognizing (qualifying) domestically produced vaccines using various types of cell substrate, and encourage exports where appropriate, principally to lower the world price of human rabies vaccines.

Advocacy and Public Information and Education

To increase awareness and enhance community participation and support, public information and education are necessary. Components of the information campaign generally have discussions on rabies as a fatal disease, its epidemiology, and its prevention and control, the disease control program in general and related national and local rabies ordinances as they support the program implementation and responsible pet ownership. With the realization of the impact of rabies in daily lives, and that pets can be a source of human infection, implementing community and school-based programs were relatively easy to roll out. Volunteerism, active engagement, and willingness to pay of people in the program stems likewise from community-based initiatives [24].

Community-based programs found all over Asia concentrate on campaigns using multimedia (television, radio, newspapers, Internet/mobile devices), display of posters and banners in strategic areas, distribution of flyers and other materials, public hearings of local ordinances and hosting of village assemblies. Some educational campaigns are often conducted at various government offices and in churches or other religious structures. Generally, celebrations like the World Rabies Day are observed to remind people of the continual threat of rabies and the importance of the program to control and eliminate the disease. School-based rabies educational programs, designed to improve awareness about rabies prevention among children are common in countries like India, Philippines, Thailand, and Vietnam. These were mostly developed and are implemented with the Ministry of Education and in coordination with the Ministries of Health and Agriculture. In the Philippines, the integration of rabies education into the school curriculum was initially developed by the Department of Health's National Rabies Control Program in 2006 [25]. Lesson plans prepared by school teachers integrated facts and figures about rabies, and lessons on responsible pet ownership. Activities for the children involve fun educational events to celebrate the bond between children and pets. The power of the youth must be harnessed. Lessons taught in interactive school programs could be brought into households and be ingrained in family values. Rabies awareness in youth and adolescents will ripple through the entire family unit, thereby ensuring

sustainable rabies interventions in future generations. The youth can proactively be involved in dog vaccination and control.

Governance, Policy, and Funding

Governments that are committed to implementing disease control programs provide the institutional framework, legislation and policies, infrastructure and logistics, human resources, and budget appropriation. The legal framework for implementing rabies prevention and control programs is already in place in most countries in Asia. National legislation defines the roles and responsibilities of the councils including dog and dog owner registration; collection of registration fees; animal population control; dog vaccination; surveillance of human and animal rabies and exposures; settlement of disputes/agreements between bite victims and dog owners; and promotion of responsible dog ownership. Funds for disease control programs are traditionally sourced from local and national governments, and international development aid. Actual implementation of intersectoral rabies control programs often requires and depends on regular budget allocation as mandated by law. International aid agencies and nonprofit organizations offer funding and technical inputs, pooling of resources, set guidelines, and standards, have monitoring and evaluation mechanisms and often act as an intermediary between donors and government.

Whole-of-Society Stakeholder Involvement

In any disease control program, wide stakeholders' involvement is critical [26]. It is important to bring together key stakeholders from business and the public sector to discuss health security and the importance of establishing public–private partnerships. Contributions from private organizations, including businesses, academe, and civil society, can be tangible and intangible. Tangible efforts are generally in the form of donations in kind or money. Intangibles such as voluntary efforts should be maximized. The intense involvement of the local communities has served as a conduit for business sectors, nongovernment organizations, academic institutions, and civil society organizations to extend their financial and technical assistance to the government. The national government agencies can sustain the standardized approaches to rabies control and elimination and promote how to start the public–private partnership that would ensure sustained intervention. Such technical and administrative conduits are essential and beneficial to all stakeholders, providing the credibility and quality assurance that is directly rooted in the day-to-day field operations.

There are numerous examples of public–private partnerships that contribute to public program implementations, support research and promote policy development in Bali, Indonesia, India, Sri Lanka, Philippines, Thailand, and Vietnam [19]. A number of rabies control programs in humans and animals have sourced funds from different sectors at different levels. The range of sources could be from the grassroots

to the corporate and people's organizations. General support to local governments given by partner organizations includes community mobilization, volunteer services, and materials donations. The business sector gives direct donations or embarks in joint ventures. The academe conducts research and offers technical inputs, voluntary services, and student manpower. The community contributes taxes, fees for service, donations, and volunteer manpower.

Field implementers and partner communities often face constraints such as high operational cost, wide regions of coverage and labor intensity. Many innovative approaches have been attempted to overcome these problems. There are numerous lessons of good practices learnt from experience. An example of a successful, sustainable community-based integrated rabies control program is the Bohol Rabies elimination program, implemented as a partnership between the provincial government, the national government line agencies (Health, Agriculture, Education, Interior, and Local Government) and a few nonprofit organizations. The project brought together educators, physicians, veterinarians, government officials, community leaders and the general public, and aligned them for coordinated effort [25]. This program produced a significant shift in rabies control, from government-dependent implementation to a community-led movement. Collateral benefits included better conditions for animal welfare, more responsible pet ownership, and improved public safety. Ownership of the program at the community level has assured more engaged field operations and sustainability. Attaining the goal of rabies control and eventual freedom from disease became a shared concern.

There are challenges though to public–private partnerships. The continued assurance of private-sourced funds depends on the effort to acquire these; thus, fund sourcing must be a full-time effort that requires a wide range of committed stakeholders. The credibility that has been established through successful local programs facilitates fund sourcing. Field experience showed that there could be disincentives to provision of external assistance including an uncertain political environment, lack of political support, and inadequate counterpart funds [7, 9].

The key to the success of a public–private partnership model is the strategic partnership among the community-based stakeholders with sound technical and operational capabilities to implement the rabies control and elimination program framework and strategic plan. The partnership ensures evidence-based and informed program planning, institutionalized organization, policies, and implementation mechanisms, the setting in place of clear performance indicators, and uninterrupted resource inputs. The key steps in project integration within the local system is the identification of key persons or technical and political champions, clear and functional feedback channels among partners (e.g., internal and external monitoring), and encouraging government empowerment and program ownership, stakeholder participation and formally defining roles and responsibilities of stakeholders through memoranda of understanding. Increased public awareness and understanding enhance willingness to pay and contribute for public good.

Rabies and the One Health Approach in Asia

Program sustainability is a critically important issue for all public health programs, but especially for resource-poor countries with limited budgets and many problems to resolve. Thus, a successful rabies prevention and control program must be built around integration and the strengthening of intersectoral and transdisciplinary collaboration and cooperation between several societal components [11, 26, 27].

The ASEAN Rabies Elimination Strategy gives particular importance to the organizational and One Health framework for rabies elimination [14]. As an example to understand better, the expansiveness of One Health challenges: In dealing with urban rabies threats, it is recognized that the best single approach is to attack the disease at its source, that is, to eliminate dog-mediated rabies. Eliminating dog rabies greatly reduces the need for postexposure human prophylaxis, at least at some point in time if the process is executed systematically. In this regard, the health sector has been at the forefront of rabies elimination programs. While this traditional principle of rabies elimination is proven to be one of the most well-based and sound of disease control strategies, in reality program implementations are confounded with complexities, resulting in more failures than successes (with only a few established and emerging exemptions). The failures have often been associated with the re-emergence of rabies after it had been temporarily eliminated in the dog population. Even areas (e.g., islands) once rabies-free have encountered emergence and endemic spread of urban rabies [8–10]. This has been the general situation for many decades. While the prescribed solution is sound and tested, i.e., elimination of rabies at-source, in the overall process, whole-of-society must deal with the complexities of prevailing urban rabies. Detailed scientific argument is not necessary to point out that poverty is a strong driver of rabies endemicity. For example, the massive proliferation of slum areas is directly proportional to rabies proliferation. The survival priorities of people dictate their health and wellbeing-seeking behaviors; obviously, food and shelter come first to those who are hungry and cold. In the same way, hungry stray dogs seek food and shelter, and the proliferations of garbage and market wastes drive these behaviors. Populations, whose general health and wellbeing are deteriorating, will be further drawn into the state of poverty. Where there are people who (must) eat dogs, there will be those who propagate and market dogs legally or illegally. There are a number of undesirable reasons why dogs are able to cross boundaries and islands. And there will always be bad governance that reciprocate bad community participation/cooperation. Such complexities are too numerous to mention all here but are at the heart of why programs fail. Very similar arguments also apply to the continued proliferation and emergence of other infectious diseases [28]. Most significantly, poverty dynamics clearly drive vulnerabilities to diseases [28–30], and these include (1) lack of adequate safe food and water; (2) lack of protection from harm such as exposure to pests, inclement weather, pollution, violence, stress, and disasters; (3) extreme social marginalization and deprivation of opportunities to earn a living, to be educated, to receive healthcare; and (4) infliction of collateral harm, especially to woman and children, the disabled and the elderly. Clearly, the determinants of infectious diseases are

multifaceted and increasingly complex [31]. Poverty reduction is central, as generally, Poverty Alleviation means Vulnerability Reduction. This has been documented in relation to the likelihood of infectious diseases emergence in impoverished community settings [32].

Good governance, involving the highest inter-ministerial central body for One Health coordination backed up by legislation and a clear mandate, budget appropriation, resources mobilization, and pilots or model programs that lead to policy development, provide optimism to implementing comprehensive operational plans that are vertical and horizontal, national and sub-national. These are important institutional drivers and enablers for a sustainable public–private partnership.

Comprehensive rabies control programs should consider combining human, financial, and material resources with other interdisciplinary disease programs to benefit from synergy and maximization of shared resources. With the guidance of OIE, FAO, and WHO, governments, donors, foundations, and other private partners should be mobilized to sustain investment in canine rabies control and eventual elimination.

Pursuing the regional goal of rabies elimination cannot be taken lightly. Sustained investment mechanism and integrative efforts must be enabled, for instance, by the designation of a specifically mandated body, e.g., a Rabies or One Health Authority directly under the Office of the President or Prime Minister. Such body could be assigned a czar (secretary or minister level) and a dedicated budget for office and resources. It should be solely focused on rabies elimination (in the meantime), and collaborate as necessary with the health, veterinary, education, environment, industry, and other sectors on clearly defined parameters and terms, with its authority maintained at all levels, i.e., national to local. The structure and mechanism for this could be legislated. Such legislation, together with the creation of the One Health authority, will remain relevant to the continuous prevention, control, and eradication of any zoonoses threat (e.g., Ebola, influenza, SARS, MERS-CoV, malaria, leptospirosis) that potentially are pandemic threats. It is important to recognize the main justification for these radical recommendations which is: any country with a prevailing human rabies threat in this modern and highly connected world is considered a hindrance to global progress.

All stakeholders are specifically drawn to the enhancement of governance. This is to ensure a sustainable approach to comprehensive capacity strengthening and broader risk reduction in the context of community resilience and regional security. The overriding objective is to advocate for continued and better targeted funding to strengthen capacities to immediately and effectively detect, prevent, and prepare for and respond to any infectious disease/zoonosis outbreaks and similar major threats. Targeted initiatives must promote broad resilience objectives, cognizant that absolute efficiency of systems, especially in relation to widespread threats, is contingent on the interdependencies of sectoral and systems approaches, and the capacity to enable strategic systems synergies.

Whole-of-government/whole-of-society coordination, involving multi-sectors within communities, is key. Therefore, rabies and zoonoses preparedness needs to be integrated into emergency and crisis response systems. The systematic involvement of even the military should be pursued.

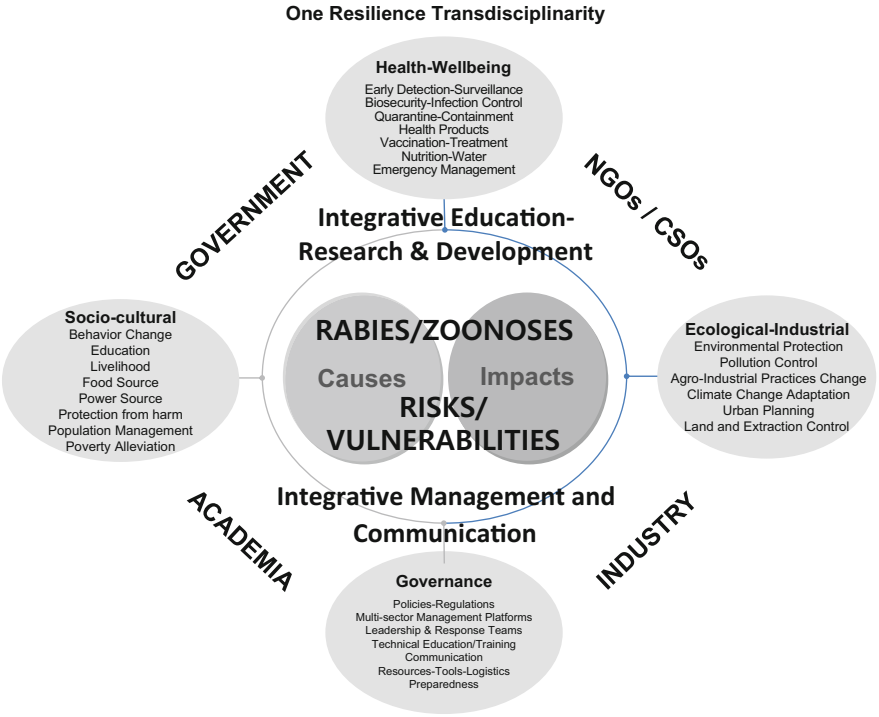

Fig. 1 Interactions among national and regional entities involved in the prevention and control of rabies and other zoonoses

The One Health Authority's core structure, functions, and capacities to plan, prepare, mitigate risk, and respond to threats through its dedicated rapid response teams should be sustainable. These must integrate into the broader whole-of-society platform and proposed "One Resilience" approach [33] to effective interactions among national and regional entities involved in the prevention and control of rabies and other zoonoses (depicted in Fig. 1), to the extent that all actors understand their roles and are enabled to effectively respond when major threats strike, so that normal operations, economic activities, and livelihood are protected and sustained.

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Passive Immunity in Rabies Prophylaxis

Charles E. Rupprecht, Mary L. Yager, and Richard H. Newhouse

Abstract

Passive immunity is a critical biological mechanism and biomedical intervention to minimize the effects of pathogens and toxins, such as in rabies and tetanus. Specifically, after the bite from a rabid animal to a person, rapid and appropriate prophylaxis virtually assures protection against a productive viral infection. Modern rabies postexposure prophylaxis in the naïve patient includes a combination of care involving prompt wound washing, active rabies vaccination, and the administration of rabies immune globulin (RIG). As a critical part of prophylaxis, passive immunity today entails the infiltration of the wound with RIG, which contains specific virus neutralizing antibodies. The source of these immune globulins may be obtained from vaccinated humans or domestic animals, such as horses. Global availability and cost are major limitations to a much wider use of RIG in reducing the disease burden among exposed persons. In the near future, RIG will remain an essential part of human rabies prevention as long as this viral zoonosis perpetuates and until such time that safe, effective, and economical alternatives are developed and are positioned for more widespread use.

Introduction

The scientific concept and clinical application of passive immunity in disease prevention is as critical a consideration today as it was more than a century ago [1–160]. Basically, passive immunity is the exchange of immune effector products (typically antibodies) from one subject (the exposed or vaccinated individual) to

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H. C. J. Ertl (ed.), *Rabies and Rabies Vaccines*,
https://doi.org/10.1007/978-3-030-21084-7_7

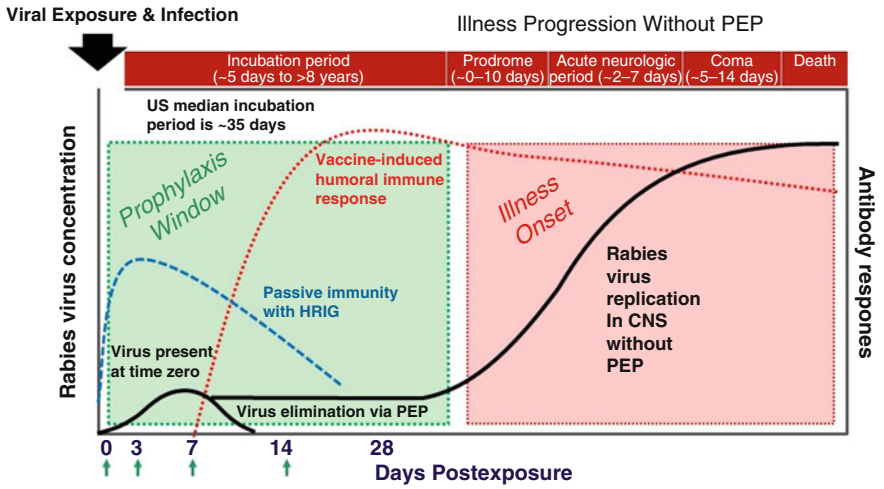

Fig. 1 Illustration of the perceived dynamics of passive and active immunity during postexposure prophylaxis (PEP) in rabies prevention compared to a productive viral infection without intervention. Adapted from Rupprecht CE, et al. MMWR Recomm Rep. 2010;59(RR-2):1–9

another, the naïve host. As a quintessential example from nature, passive maternal immunity occurs via the transplacental transfer of antibodies from the dam to the fetus or by the consumption of colostrum by the newborn, depending upon species [1, 2]. The general biomedical thesis of applied passive immunity, directed to toxins (e.g., antitoxins, such as tetanus toxoid) and against infectious disease pathogens, dates back to the late nineteenth century, with the pioneering work exemplified by von Behring, Kitasato, Ehrlich, and others, even before the biochemistry, genesis, and action of antibodies was well defined [3–8]. While most of the history of immunization against rabies focuses upon the now classical model of active vaccination after Pasteur's notable achievements, use of immune serum, alone or in concert with vaccine, is clearly as old in concept. Later in the twentieth century, post-World War II, the overall use of immune serum in passive immunization as a whole was advanced by Cohn's method of purifying antibodies from plasma by ethanol fractionation [9].

Passive immunity is a critical part of the medical repertoire in human rabies prevention [10]. Following a lyssavirus exposure, the primary rationale for passive immunity is framed upon the need to supply a source of specific, preformed virus-neutralizing antibodies (VNA) during postexposure prophylaxis (PEP), in the time before active immunity develops after vaccination (Fig. 1). Rabies VNA via passive immunization may be detected in the serum within 24 h of administration (Fig. 2). Passively delivered antibodies remain present in the intervening days to weeks before active immunity from vaccination ensues. In cases of transdermal or mucosal exposure to rabies virus (RABV), rabies immune globulin (RIG) is a critical biologic for successful PEP, especially after severe exposures, such as bites to the face and

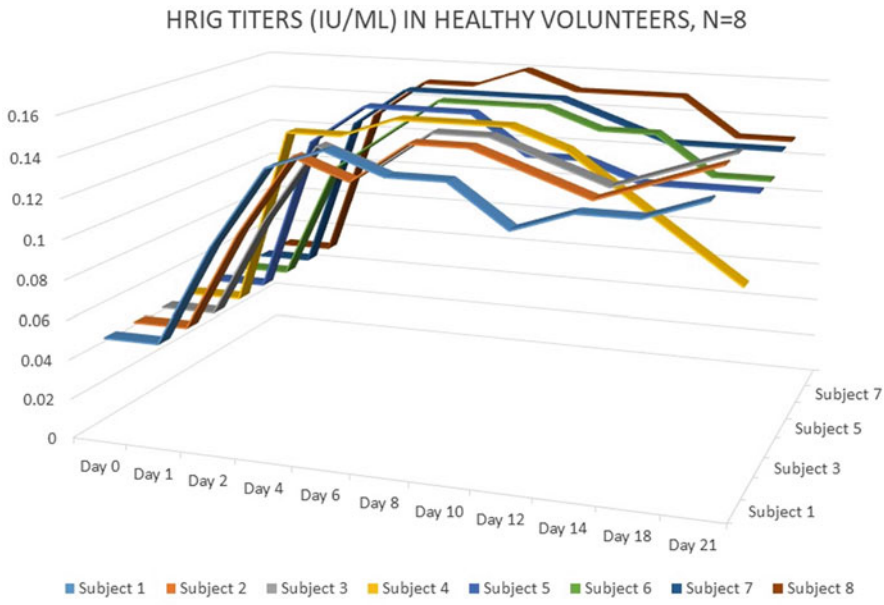

Fig. 2 Analysis of rabies virus neutralizing antibodies after IM administration of human rabies immune globulin (HRIG) alone at 20 IU/kg to healthy human subjects (AHA Inc., unpublished data)

head, where time is of the essence to abrogate a productive viral infection [10–17]. Moreover, together with thorough wound washing, application of RIG may be the only source of successful intervention in cases of severe immune compromise, where little to no active induction of VNA may occur [13]. In addition, although utilized primarily for prevention prior to the advent of illness, RIG may be a critical immune component for consideration of experimental treatment of clinical rabies cases, taking into account the necessity for facilitated transport across the blood–brain barrier [18].

The dose of RIG administered is a compromise between passive maximum efficacy in the neutralization of RABV virions and the potential resulting interference with active immunity from vaccination [19–30]. In addition, the source of RIG used during PEP is a choice as to source, which may be heterologous from vaccinated animals, typically equine (ERIG), or homologous, from vaccinated humans (HRIG). Such overt benefits of RIG were substantiated gradually over the past century, although multiple limitations are also evident (Table 1). Given the critical utility of passive immunity during PEP, suitable alternatives to the use of RIG in humans have been proposed and under consideration for at least 40 years, some of which are only coming to fruition today ([31]; https://www.seruminstitute.com/product_ind_rabishield.php).

The objective of this chapter is to provide an overview of the history, utility, limitations, and alternatives related to passive immunization in rabies prevention.

Table 1 Potential benefits and limitations of rabies immune globulin

Utilities	Drawbacks
Proven historical antecedent for use after infection	Cost
Broad cross-reactivity across viral taxa, including all phylogroup 1 lyssaviruses	Global availability to persons at risk
Commercial production in Africa, the Americas, and Eurasia	Lower antigen-specific activity
Only option for the severely immune-compromised patient	Potential for lot-to-lot variation in potency and storage
Can be produced in multiple warm-blooded species	Biosafety of blood-derived products and foreign proteins
Reasonably long half-life to bridge the temporal delay between infection and active induction of immunity from vaccination	Large volumes administered based upon individual body mass
Minimal adverse events	Potential interference with active immunity
Ability to enrich for IgG isotypes and subtypes	Ethical and cultural concerns to blood product use

History

Conceptually, before acceptance of the "germ" theory of disease, after the bite from a rabid animal the ensuing lesion was considered the probable portal of entry of the "poison" or "slimy liquid" leading to illness and death. Consequently, the current practice of local RIG infiltration is a natural scientific progression from the attention upon wound treatment, including such historical examples of copious irrigation, amputation, cauterization, envenomation and application of acids, chili powders, or other noxious substances over the centuries [32]. Alternatively, during the late nineteenth century to the mid-twentieth century, multiple investigators provided ample evidence on the use of anti-rabies sera [33–46], in a variety of animal models (Table 2). These studies led to several generalizations: rabies mortality increased as anti-serum was diluted, suggesting a dose response; survivorship improved when administration of anti-serum occurred very soon after viral infection; local infiltration of anti-RABV serum near the site of viral inoculation was superior to distant application; antibody use alone did not confer long-term immunity; and protection appeared best when passive immunization was combined with vaccine. Such observations were corroborated into the later twentieth and twenty-first centuries.

If such inferences began at about the same time as Pasteurian vaccination, why was the thesis of passive immunity and "sero-protection" so slow for medical acceptance until near the end of the twentieth century? Explanations are multiple: animal sources and quality varied in early research; numbers of subjects were usually small; experiments were not well controlled; repeatability was limited; standardization was less than ideal; antibody demonstration remained poorly defined; potency measurements were lacking; technical methods for anti-serum

Table 2 Examples of historical anti-rabies serum use in animal models

Subject	Source	Findings	References
Rabbit	Whole blood from hyperimmunized dogs	Transfer of passive RABV protection	[33]
In vitro use	Sera from vaccinated rabbits	Apparent neutralizing activity against RABV	[34]
Rabbit	Serum from vaccinated sheep	Resistant to RABV challenge	[35]
Dog	Sheep antirabies serum mixed with live virus	Apparent protection against RABV for at least 1 year	[36]
Mouse	Rabies anti-sera from vaccinated horses, donkeys, sheep, dogs, or geese	Protection against subcutaneous RABV challenge even if delayed for several days	[37]
Rabbit	Anti-sera from vaccinated rabbits, sheep, horses, or dogs	Survival after RABV challenge in the anterior eye chamber	[38]
Guinea pig	Anti-sera from sheep	Protection when administered simultaneously at the site of RABV infection	[39]
Mouse	Anti-rabies serum from goats	Passive immunization could interfere with RABV vaccination	[40]
Mouse	Intraperitoneal administration of serum dilutions after challenge infection	Suggested a method for potency determination of anti-RABV serum	[41]
Guinea pig	Hyperimmunized rabbit sera	RABV protection in proportion to serum dose	[42]
Guinea pig	Equine anti-rabies serum	Superior protection over chemical viricides, even if delayed for 24 h after RABV infection	[43]
Guinea pig	Dried equine anti-rabies IgG	Decreased protection as the administration was delayed after infection	[44]
Syrian hamster	Concentrated rabbit or sheep anti-rabies sera	Correlation with RABV challenge dose, globulin concentration, and delay before administration	[45]
Guinea pig	Anti-rabies equine IgG	Best results with wound cleansing and serum infiltration against RABV	[46]

production remained less than optimum; the extension from animal models alone was unconvincing without ample proof in *Homo sapiens*; and safety concerns abounded. Once methods of such inquiry were better refined and resulting products improved, a gradual reassessment began [47–56], as usage in humans progressed beyond the anecdotal and small scale to larger clinical trials (Table 3). In general, these studies in toto demonstrated the utility of passive immunity in concert with vaccination, especially after severe RABV exposure incidents.

Table 3 Selected prominent cases of rabies anti-serum use in humans

Incident	Application	Outcome	References
A dozen people severely are bitten by a rabid wolf	Whole blood from immunized people or dogs plus Pasteur vaccination	All 12 persons survived, while one untreated person succumbed	[47]
More than 300 severely bitten persons over a period of 4 years	All given a combined Pasteur vaccination and serum protocol	All persons survived	[48]
More than 200 severely exposed persons over the course of a year	Anti-rabies horse serum administered SC plus vaccination	All persons survived	[49]
Laboratory or bite exposures ($n = 29$) in the United States	Administered sheep anti-rabies globulin IM at 1 mL per kg plus vaccine	All persons survived	[50]
Serological responses in nonexposed adult volunteers	Given hyperimmune serum and/or vaccine	Antibody appeared a day after serum administration and persisted for at least 21 days whereas it took at least 10 days to appear after vaccination alone	[51]
Seventeen individuals exposed severely to wolf bite in Iran	Some persons received vaccine only whereas the rest received vaccine plus anti-rabies serum	Three of five persons given vaccine only died while 11 of 12 persons administered vaccine plus serum survived	[52]
Human exposures to virulent strains in Vietnam	Intensive course of vaccination combined with purified and concentrated anti-rabies serum	Complete protection was observed after the use of combined prophylaxis	[53]
Humans are bitten by wolves or domestic animals in the former USSR	Thirty-six persons were given equine anti-rabies globulin plus vaccine while 28 others were given vaccine only	All persons given combined prophylaxis survived, while 11 of those given vaccine only succumbed	[54]
Forty-four severely bitten persons in Iran	Administered HDCV vaccine plus rabies-immune serum	All persons survived	[55]
Humans are bitten severely by rabid animals in China	Given primary hamster kidney cell rabies vaccine and purified equine antirabies serum	All persons survived if administered within 3 days of exposure, whereas others died	[56]

Proposed Action of Antibodies During Passive Immunity

The dynamics of RABV infection and immunity are complex. Following a bite from a rabid animal, virions in the saliva are deposited into the wound, access cellular receptors, replicate slowly, evade innate and adaptive immune responses, and begin a centripetal tropism toward the central nervous system from the periphery [57]. Natural immunity to rabies is poorly understood, but doubtless involves a combination of innate and adaptive responses that vary by taxa [58–70]. Acquired immunity to RABV in reservoir mammals may be important in the initial development and later passive transfer of maternal VNA from the dam to the fetus or neonate by transplacental and colostral delivery of immune globulins. Waning passive immunity in young of the year may explain in part the seasonal occurrence of disease outbreaks [66, 67]. In exposed or vaccinated domestic and wild species, passive maternal immunity may interfere with the development of a robust immune response in young animals [71–77]. This observation led to the recommendation of waiting 3–4 months before initiation of vaccination in the juvenile animal [78].

Interruption of a productive viral infection during human PEP is via the combined action of RIG and vaccine. The rabies virions induce specific VNA responses against the RABV glycoprotein, during vaccination and during the production of RIG [79]. Several different mechanisms of action provided by VNA have been suggested to include steric hindrance by attachment to virions and interference with receptor attachment, as well as inhibition of the intra-endosomal acid-catalyzed fusion step needed for viral un-coating, initiation of complement-dependent lysis of RABV-infected cells and mediation of antibody-dependent cellular cytotoxicity [80–84]. The majority of VNAs induced by vaccination will be directed primarily against RABV and phylogroup I lyssaviruses, including Australian, Bokeloh and European bat lyssaviruses and Duvenhage, among others [85]. In theory, some vaccine recipients may develop cross-reactive immunity against more genetically disparate lyssaviruses [86]. However, during production, lots of RIG are only screened routinely against a laboratory strain of RABV, such as CVS, hence the breadth of lyssavirus reactivity in commercial products is not known.

Equine Antibodies for Passive Immunization: An Outline of ERIG Production

Antibody production may occur in the same species as the intended user (i.e., human for human, HRIG), or heterologous, by production in one species but used in another (i.e., horse for human, ERIG). Some of the first applications of antibodies were heterologous preparations, simply crude sera from vaccinated animals [87]. Protocols evolved over time and outcomes depended in part upon the donor species, the type and potency of the vaccine used, the inclusion of adjuvant and the schedules of immunization [88]. Although several types of mammals were used, larger bodied animals, such as small ruminants and horses, were often employed for production due to their body size and ability for multiple bleedings by plasmapheresis

Table 4 Key equine rabies immune globulin production steps (as described, [94])

Recruitment of healthy donor animals
Serial rabies vaccination of horses
Individual serum sample testing for potency determination
Hyperimmune donor serum collection by plasmapheresis
Plasma pooling and dilution
Plasma purification via digestion and thermocoagulation
Hydrolysis into Fc and F(ab')2 fragments
Precipitation of non-IgG proteins
Removal of precipitates by ultrafiltration
Blending and dilutions
Sterile filtration
Final bulk filling
Formulation
Product release after acceptable purity, potency, safety, and stability testing

[89]. Unfortunately, not only did these equine preparations vary greatly in quality, but also due to their nature as heterologous proteins, they also carried the risk of adverse events, such as serum sickness or anaphylaxis [90, 91]. Purification can be used to maximize antibody potency and minimize the risk for antigenic sensitization [92]. Over the past several decades, great improvements have occurred in the potency, safety, and effectiveness of ERIG [15, 93]. As one example of some of the current methods used for ERIG production, a generic protocol utilized by the Thai Red Cross [94] is illustrated in Table 4. Besides the risk of potential adverse events from heterologous products, cultural norms, religious beliefs, and animal welfare concerns may limit a more widespread use of commercial ERIG, particularly in the developing world.

Human Antibodies for Passive Immunization: An Overview of HRIG Production

Historically, before improved purification techniques, the use of heterologous equine anti-rabies serum resulted in the development of serum sickness in ~16–46% of recipients [95]. Due to such an unacceptably high proportion of adverse events, efforts began to prepare anti-rabies immune globulin of human origin [96]. However, even in the early days of consideration, production of HRIG was neither simple nor inexpensive. For example, to meet the expected minimum annual demand for HRIG in the United States alone during the 1970s, between 500 and 900 vaccinated human volunteers would have to donate blood every 2 weeks, to administer to one average-sized person during PEP, at an estimated cost of ~$1000 USD per delivery [96]. Today, similar to ERIG, the manufacture of HRIG goes through many uniform steps of production and testing regarding concentration upon safety, purity, and

potency, with the obvious exception of digestion and formation into F(ab') 2 fragments [97].

Human blood plasma used for production of HRIG is obtained from individual donors. Healthy volunteers are qualified for entry into a plasma donation program and screened for acceptance by a rigidly defined set of criteria of reactogenicity against licensed in vitro markers for known blood-borne pathogens and potentially emerging agents, which include viral hepatitis, human immunodeficiency virus Type I, syphilis, etc. [98–110]. Other tests may be needed as defined by national licensing approvals. A questionnaire is also required for each candidate, usually given by the attending licensed physician at the collection center, to include "life-style" issues (such as recent tattoos or piercings, and sexual activity), age and weight (which will define the total amount of plasma allowed to be collected at the time of each particular blood draw, from ~600 to 800 mL). If the candidate qualifies for the program, plasmapheresis is allowed to be performed. These collections can be made as often as two times per week without known adverse consequences. Each plasma collection is subject to a rigid protocol, as disease markers during the pre-enrollment and early, pre-symptomatic stages of pathogen infection may escape the detection of current in vitro assays. During the GMP system, safety of RIG from contamination by adventitious agents may include treatment with solvent/detergents, pasteurization, and nano-filtration.

During RIG development, donors are hyperimmunized with licensed cell culture RABV vaccine (e.g., HDCV, PCEC, and PVRV) to produce high concentrations of VNA. This requires routine primary pre-exposure vaccination and booster doses with potent vaccine (if permitted by a standard operating procedure), as VNA decline gradually. Nervous tissue vaccines are no longer recommended and are rare in global use today and should not be considered for routine human immunization for the production of HRIG, given readily available tissue culture alternatives. High responders to such vaccination are retained and boosted ad hoc every few months, if permissible and available for continued plasmapheresis. As rabies vaccination is often a requirement for entry to veterinary school, recruitment programs have been successful with access to such willing students, who may be available for 3–4 years, as opposed to random volunteers. Strict regulatory limits are set on the maximum volume obtained per donor, such that it would be unusual to obtain more than 0.5 L of plasma from a given donor per year.

The individual hyperimmune plasma samples obtained from donors are tested prior to consideration for use in manufacture of HRIG, to ensure a minimum level of VNA, or potency, meets defined specifications [97]. After collection at the plasma collection facility, each unit is frozen at or below −20 °C and monitored to ensure the temperature does not exceed that value. If the units thaw above −20 °C, all are rejected for use in the HRIG program. This temperature is maintained throughout storage and shipment, until receipt at the HRIG production facility. If thawing outside of this range occurs, all units are rejected as starting materials for the subsequent processing. Samples of the plasma pool are tested for rabies VNA to verify acceptable potency for further production. The HRIG production facility then thaws and pools the units meeting the VNA requirements for that production run.

Following pooling, the plasma is fractionated, by separation and collection of target constituent proteins within the plasma. Separation occurs by precipitating desired proteins out of solution by manipulation of temperature, pH, and alcohol concentration. Proteins are harvested by depth filtration and centrifugation. The final fractionation product is collected via centrifugation and frozen. After resuspension, the material goes through a clarification filtration and is diafiltered to remove alcohol. Thereafter, tri-n-butyl phosphate (TNBP) and sodium cholate are added to the solution and the product is incubated at an elevated temperature, as a viral inactivation step. After incubation, the TNBP/sodium cholate is precipitated out of solution by adjusting pH, and removed by a clarification filtration. The product is again diafiltered against glycine and ultrafiltered to a target protein concentration. Sterile filtration is performed to make an initial master bulk. The potency of the initial master bulk is adjusted with portions of other sterile bulks prepared from normal, non-hyperimmune samples, to achieve a target potency prior to filling the finished product. The sterile bulk is filled aseptically into vials (e.g., 2 mL and 10 mL) and incubated at 20–27 °C for 21–28 days. Thereafter, the product is stored at 2–8 °C, pending quality control testing, packaging, and distribution. The shelf life may exceed 2 years when stored appropriately.

A rabies VNA potency test is performed at several steps during the production process and on the finished HRIG lot to ensure that the product meets the potency specification for lot release and throughout its intended shelf life [97]. Most licensed HRIG products have a minimum potency of at least 150 IU/ml contained in a 10 mL vial intended for adult use (pediatric use vials may be at a smaller volume of ~2 mL). With modern potent rabies vaccines, on average, a group of 20 healthy vaccinated donors, whom each respond reliably with a minimum VNA determination of ~ 10 IU/ml, providing plasma routinely over the course of one half year, might supply the necessary raw material for at least 1000 vials of HRIG. Units of lower than ideal VNA content could be considered for blending with high respondent pools or used for other standard human immune globulin products.

Potency Determination of VNA in RIG for Passive Immunization

The dose of RIG used for PEP was eventually determined in preclinical and clinical studies as the amount of biologic that resulted in detection of VNA yet did not interfere totally with active immunization [19–30]. Hence, 20 IU/kg and 40 IU/kg were selected as a compromise for the dose of homologous and heterologous products, respectively. Previously, serum or RIG potency was determined by in vivo methods, such as the mouse neutralization test [111]. Today, the relative content of specific RABV VNA in RIG, used to define potency, is determined by cell culture methods, such as the rapid fluorescent focus inhibition test (RFFIT), as described [112]. Briefly, serial dilutions of RIG are incubated with a working dilution of RABV, such as the challenge virus standard (CVS-11) strain, although other viral strains have been used. A Standard Rabies Immune Globulin preparation (e.g., R-3, assigned a value of 59 IU/ml) is diluted to a working dilution of 2 IU/ml,

tested in parallel with the RIG. After incubation of the RABV-serum dilutions, cultured murine neuroblastoma cells (MNA) or baby hamster kidney (BHK) cells are added in chambered slides and incubated for 18–22 h at ~35–37 °C with 0.5–5% controlled CO_2. Following incubation, slides are fixed in acetone and stained with fluorescein (FITC)-labeled anti-RABV antibody conjugate for 30 min at 37 °C. The slides are rinsed with buffer, and dried at room temperature in preparation for microscopic analysis. Viral infection of the MNA or BHK cells is identified microscopically by observation of intracytoplasmic apple green inclusions. Twenty fields are examined for fluorescing foci or clusters of infected cells in each of the chambers on the slide. A given field is considered positive for RABV infection if one or more fluorescing foci are observed. The total number of positive fields, out of twenty fields examined, is determined for each product/reference dilution level. The endpoint titer is determined by the dilution at which 50% neutralization is achieved (50% reduction in the fields positive for RABV infection as compared to controls). Mathematical interpolation of the 50% neutralization endpoint titer is performed by the Reed-Muench method. The titer of the RIG product is converted to a potency value in terms of IU/ml by calibration against the endpoint titer of the standard tested on the same assay. To mitigate potential variability that is inherent in cell-based assays, the RIG is tested using replicate dilution series on each assay, with at least two independent tests performed. The final potency is the mean of test determinations that meet a defined CV limit criterion for intermediate precision and repeatability established during assay validation. The VNA titer testing may occur serially in animal or human donor serum samples, pools of produced plasma and final RIG product.

Utility of RIG in Passive Immunization During PEP

In developed countries, virtually all individuals who are known or thought to be exposed to RABV obtain PEP. In a country such as the United States, approximately 16,000–60,000 persons per year may receive PEP (http://www.immunize.org/askexperts/experts_rab.asp). With the advent of licensed HRIG and cell culture vaccines during the 1970s, no PEP failures have been reported in the United States to date. Given the obvious benefits of modern PEP, why do human rabies cases still occur? Health disparities are responsible for most of the tens of thousands of human rabies cases, occurring disproportionally in developing countries [113]. Most humans who succumb to rabies receive no PEP at all. This may occur because of a lack of community education on RABV exposure and prevention. The majority of exposed persons who do seek and obtain PEP may only receive vaccine. Less than 2–5% of patients may be given RIG. This unacceptably low rate of RIG use is a combination of neglect, local availability, and cost of goods and services. The ability to pay for PEP should never be detrimental to receipt of life-saving biologics.

When exposure is recognized and PEP is prompt and proper, survivorship is virtually assured, even in extreme circumstances. The outcome between infection

and immunity is due in part to the viral variant, exposure route, infectious dose and severity of the event, coupled with host characteristics (including immune compromise) and delivery of appropriate health care. Cases of so-called PEP failures must differentiate among factors related to the biologics, the patient and the provider. Unlike historical precedents, the quality and stability of modern biologics are superb, if the cold chain is maintained. Counterfeit products have been reported in a few instances, but such incidents are rare. Severe and multiple bites to the face and head and highly innervated extremities such as the hands present some of the largest challenges, especially when wounds are not cleaned immediately, and the patient presents for PEP days to weeks after a bite. Major medical misadventures include inadequate wound cleansing and debridement, lack of local RIG infiltration and suturing of wounds before RIG administration [114–122]. Apparent human PEP failures are attributed primarily to rabies virus infection, as opposed to a lack of reactivity against other viruses in the Genus. To date, there have been no documented human PEP failures due to Phylogroup II and other genetically diverse lyssaviruses. However, surveillance is much less than ideal in those localities where risk may be greatest [123, 124].

Adverse Events in the Use of RIG During Passive Immunization

Considering the high case fatality of rabies, there are no true contraindications to the use of RIG in the naïve person during PEP [11, 12, 14]. No serious adverse or allergic reactions have been reported as causal in association with ERIG or HRIG use [125–127]. Most local reactions, such as complaints of pain, or tenderness at the injection site, were mild or moderate, as were transient systemic effects such as fever, chills, and headache, among others. Known sensitivity to any of the components of the biologics involved would entail considerate use of an alternative product, if available and medical intervention if any adverse events were noted. There are a number of potential risks that may occur with any RIG, that include transmission of infectious agents, hypersensitivity, and thrombotic or hemolytic events (https://www.drugs.com/cdi/hyperrab-s-d.html; https://www.drugs.com/cdi/imogam-rabies-ht.html; https://www.drugs.com/cons/kedrab.html). However, unwarranted fear of such adverse events and hesitation to receive PEP in RABV-exposed patients may lead to extremely unfortunate outcomes [128].

In previously vaccinated persons, RIG is not indicated, as priming has already occurred in pre-exposure or PEP and booster administration of vaccine will cause a rapid anamnestic response. The use of RIG may cause interference with active immunization, which is a reason that dosing should not be above recommendations [129]. Despite historical reports of anaphylaxis from the use of horse serum, there are no scientific grounds for performing a skin test before administering a heterologous product, such as ERIG, because testing does not predict adverse reactions and

RIG should be administered to the exposed person, regardless of the test results [130].

Potential Alternatives to Current Passive Immunity Practices

A better understanding of the dynamics of VNA in viral clearance would enhance opportunities for passive immunization, based in part upon expanded practice and future products. Restriction on the availability of RIG is one of the greatest limitations to more widespread use, with a need for improved production and supply. Beyond improvements to risk assessment and patient triage, immediate resolution of critical supply issues could be lessened by concentrating upon local infiltration of wounds alone, rather than application of the total calculated systemic dose administered at an intramuscular site [131]. Disparate clinical acumen may limit such practices to all but the most experienced health care providers. Rather than local muscle injection when wounds are not readily identified, some have espoused the IV route as a means to improve the circulation of Ig molecules throughout the body [132]. Fears of anaphylaxis may restrict application of such alternative routes of delivery. Greater concentration of HRIG to 300 IU/ml from the standard of 150 IU/ml may ease concerns over volumes and compartment syndrome, as well as limit the necessity for multiple injections, such as in pediatric patients and facial lesions (https://www.prnewswire.com/news-releases/grifols-hyperrab-rabies-immune-globulin-human-300-iuml-receives-fda-approval-to-treat-patients-exposed-to-rabies-virus-infection-300594182.html).

Expanded biodiversity considerations could be applicable to greater RIG availability for passive immunization needs. Beyond humans and horses, relatively few other species have been considered for RIG production, such as avian egg use for IgY or camelids for "nanobodies" [133, 134]. Monoclonal antibodies (mabs) as an alternative to RIG have been in development for decades, produced via murine or human hybridomas [135–143]. "Plantibodies" have also been considered [144, 145]. Approvals of the first human monoclonal antibody for PEP may begin to herald the application of such technology to public health practice (www.seruminstitute.com/product_ind_rabishield.php).

Although passive immunization in rabies PEP has focused upon VNA, alternatives include interferon and interferon inducers, some of which have demonstrated efficacy in experimental animal models against virulent rabies virus challenge [146–156]. Experimental use of hybridomas and immune effector cells has also been considered, but beyond theoretical applications, clinical practicality may be limited [31, 157]. Technical improvements in greater vaccine potency, faster active elicitation of VNA and induction of innate immunity may obviate the need for any RIG or mabs during PEP in the shape of things to come (Table 5).

Table 5 Program actions for reducing or obviating the need for a RIG in PEP

Practical community minimization of human exposures to suspect animals
Enhanced public health and responsible pet owner education
Improved use of PPE in the laboratory and the field
Focused pre-exposure vaccination of human populations at risk
Mandatory dog and cat vaccination (as well as other relevant domestic or exotic species)
Enforced leash legislation
Increased access to domestic animal spay and neuter services for population management
Performance of proper risk assessments for the animal bitten patient
Utilization of animal control authorities in the capture of rabies suspect animals
Adequate holding facilities for animal observation, quarantine, euthanasia, and necropsy
Adoption of rapid, sensitive, and specific diagnostic tests of the suspect animal
Actualization of cost-effective alternative sources to RIG, such as monoclonal antibody cocktails
Applied research on vaccine potency, adjuvants, or alternates to passive antibody necessity (such as cytokine inducers)
Modern oral vaccination of mesocarnivores to reduce the overall disease burden in nature
Humane bat exclusion from human dwellings to lessen the chance of an exposure

Unresolved Issues in Passive Immunity

In theory, human PEP is fairly straight forward regarding administration and predictable in outcome when the diagnosis of the suspect animal is rapid, the patient's lesions are washed immediately with ample soap and water, the first dose of cell culture rabies vaccine is administered on the same day and RIG is infiltrated into and around the wounds at the correct dosage per current guidelines [11–14]. In practice, many deviations may occur and troubling questions abound [16]. For example, in shortage situations, is it ethical to reserve the use of RIG (such as for only multiple bite exposures)? With the use of ERIG products (as less than intact Ig molecules having shorter half-lives) is it ever warranted to administer more than a single dose (such as in a severe bite to the face and head)? How should a maximum dose of RIG be calculated (such as in a morbidly obese patient)? If only RIG is available, should it be used alone before vaccination (such as when vaccine supply is absent)? What total volume of RIG should be administered to the tip of an appendage (such as from a severe cat bite) to remain efficacious but to avoid compartment syndrome? Once a category III bite exposure has been defined more than 6 months to a year after exposure (such as in exit interviews of military personnel in canine rabies enzootic regions), could PEP with vaccine only suffice? When a mucosal exposure involves the eye (as in a splash incident), would corneal washes or intraocular administration make sense? If the probability of exposure cannot be ruled out, but a bite is unrecognized (such as in certain bat situations), should RIG be administered or vaccine only applied? Should RIG ever be used for indirect, non-bite exposures (such as a person who rescues their pet from a suspect attacking animal)? Within a One Health context, will recommendations for passive immunity ever be extended

routinely to the naïve animal exposed to RABV? Many of these and other conundrums will remain unresolved as regard an evidence-based solution related to viral pathobiology, host immunity, product inserts, epidemiological insights, or health recommendations. Dialogue with experienced clinicians and informed public health professionals may help to ease such concerns as they arise on a case by case basis [158].

Many such nagging problems in the use of RIG would be minimal if cost and availability were irrelevant. Unfortunately, despite clear life-saving benefits, RIG has been underutilized for the patients who require modern PEP the most. At a starting base, the transdisciplinary application of multiple modalities may be used to minimize the need or replace the use of RIG in a more holistic One Health context (Table 5). Clearly, discussions over the proper and timely administration of RIG magnify a failure of the basic public health and veterinary infrastructure, because the individual is already exposed to a virulent pathogen from an affected subject, regardless of communications, education, or animal vaccination.

Although possible, the global elimination of human rabies transmitted by dogs (GEHRD) through mass vaccination will not be simple, rapid, or inexpensive [159]. Beyond the biomedical variables, a variety of anthropological, economic, political, and social barriers may need to be considered for more effective resolution [160]. Regardless, rabies is not a disease considered for eradication. At a minimum, human exposures will continue from wildlife and cross-species transmission to unvaccinated domestic animals, such as cats. Hence, prophylaxis will be critical as a mainstay in public health and veterinary prevention of cases. Unfortunately, despite a century of historical intent, routine utilization of functional passive immunity during PEP of exposed patients in the developing world remains at a minimum, as for other plasma products [9, 98, 102, 104, 106, 108]. Given the pharmaceutical challenges and regulatory hurdles in many such countries, it remains to be seen if additional safe and potent RIG products (or alternates?) will be produced, where they may occur (as needed most in Asia and Africa?), when this may transpire (such as in line with the intended 2030 GEHRD?), what predictable quantity will be delivered (at an affordable price?) and who will be the main local and regional champions and suppliers in the near future (to minimize dependence upon external Western producers)? Answers to such questions will help to better refine the critical role of passive immunization in both developed and developing countries alike for rabies prevention, in the wake of new plans, procedures, products, programs, and protocols [160–165].

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Rabies Little Virus Against Powerful Innate Immunity

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Abstract

The innate immune system provides a very rapid defense mechanism against invading pathogens unlike adaptive immune responses, which require several days for cells to expand and differentiate till they are fit to assume effector function. Innate responses through the recognition of pathogen-associated molecular patterns by pathogen recognition receptors trigger the initial inflammatory reactions to pathogens. Rabies virus induces innate immune responses including cellular responses and production of antiviral cytokines such as type I interferons, which slow down spread of the virus. In turn rabies virus has evolved to circumvent innate immune responses.

Introduction

The major coordinator of innate immunity to rabies virus (RABV) is the Type I interferon (IFN) family of cytokines. These proteins, which are upregulated in response to viral molecular patterns detected in the cytoplasm, act in an auto- and paracrine fashion to stimulate the expression of hundreds of interferon-stimulated genes (ISGs) with diverse and often unknown antiviral functions [1]. The signaling pathway leading from virus detection, to IFN upregulation, to IFN detection by cells,

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H. C. J. Ertl (ed.), *Rabies and Rabies Vaccines*,
https://doi.org/10.1007/978-3-030-21084-7_8

and finally to ISG upregulation is disrupted at every step by different viruses, including RABV. The best understood of RABV's countermeasures to the IFN system is the phosphoprotein (RABV-P), which inhibits both IFN and ISG upregulation via two distinct mechanisms (reviewed in [2]). RABV also uses more generalized approaches to avoiding detection, such as sequestering nascent particles in a phase-separated inclusion body, and encapsidating its potentially immunostimulatory RNA genome in nucleoprotein as it is produced.

Despite the central role of IFN, innate immunity to RABV does not completely depend on a functional IFN system. Mice deficient in the IFN-alpha receptor (IFNAR), which is the only cellular receptor to type I IFN and therefore an absolute requirement for a canonical IFN response, are occasionally able to survive experimental RABV infections, despite higher mortality [3]. This review explores both the interferon-dependent and -independent mechanisms of RABV innate immunity, and also the various countermeasures employed by RABV to undermine these mechanisms. Those aspects of RABV innate immunity that are distinct from related viruses such as vesicular stomatitis virus (VSV) are emphasized.

Detection and Response to Viral Molecular Patterns

The IFN response, like all immune signaling pathways, starts with the detection of nonself molecular patterns. The foremost pattern recognition receptors for RNA viruses are the cytoplasmic Rig-I-like receptors (RLRs), retinoic acid-inducible gene I (RIG-I), melanoma differentiation antigen (MDA5), and Laboratory of Genetics and Physiology 2 (Lgp2). RLRs are members of the DEAD-box RNA helicase family, which impact a number of other cellular processes including RNA binding and the alteration of RNA secondary structure. Additionally, RLRs are homologs of the Dicer-related helicase (DRH-1) responsible for antiviral RNA interference (RNAi) in nematodes [4, 5]. Downstream signaling depends on N-terminal caspase activation and recruitment domain (CARD), and a central ATP-dependent helicase domain, which recognizes RNA ligands [6]. The CARD domain is missing in Lgp2, making it likely that Lgp2 acts as a negative regulator of IFN activation [7, 8]. RIG-I was the first RLR to be implicated in the IFN response [9]. Early studies suggested that RIG-I recognized dsRNA, although the precise RNA ligand remained controversial for some time (reviewed in [10]). RIG-I's connection with RABV was established by evidence that RABV-related IFN stimulation could be enhanced in vitro by RIG-I overexpression and suppressed by expression of a dominant-negative RIG-I mutant [11]. Transfections of various RABV-related RNAs—including the single-stranded RABV genome, RABV leader RNA (leRNA), and bulk RNA from RABV infected cells—stimulate IFN production in vitro, but cease to do so after the enzymatic removal of the 5′-triphosphates [11]. This evidence suggested that in addition to sensing phosphorylated dsRNA, RIG-I senses the single-stranded genome or leader RNA, a result supported by other studies [12]. With more detailed structural examinations of RIG-I, it was found that nonself RNA is detected based on the 5′-triphosphate *and* the lack of methylation at the 2′-O position of the first

nucleotide [13, 14]. This disagreement over the RIG-I ligand stemmed from the fact that 5′-triphosphate RNA is always 2′-O unmethylated when it is first produced by cells and viruses [15].

MDA5, which is thought to recognize blunt-ended dsRNA exclusively, was first considered relevant primarily to positive-stranded picornavirus infections [16]. However, in vivo experiments suggest a more complex functional overlap between the two major virus-sensing RLRs. For instance, ISGs were induced by various RNA viruses in both RIG-I and MDA5 knockout mice [17]. In a RIG-I knockout mouse BMDCs infected with RABV, IFN induction was delayed, but not fully abrogated [18]. The RLR inhibitor Lgp2 plays an ambiguous role: overexpression of Lgp2 inhibited IFN expression after RABV infection in vitro, but paradoxically reduced RABV mortality in vivo [19]. Furthermore, it is unknown which virus-derived RNAs are detected in particular infections. For most RNA viruses including RABV, virus-encoded RNA capping activity eliminates 5′ triphosphates and 2′-O unmethylated RNA early during RNA production [20]. Genomic and antigenomic RABV RNA are not capped or methylated at the 5′ end but are quickly encapsidated in nucleoprotein which may shield them from detection from RIG-I [21, 22]. Therefore, the physiological RLR ligand for RABV seems most likely to be either leader or trailer RNAs, which are uncapped and not encapsidated, or aberrant replicative RNAs such as defective interfering (DI) RNAs [1].

Following the detection of RABV RNA, RIG-I stimulates IFN production by a characteristic signaling pathway. RNA binding brings the CARD domain of RIG-I into contact with the CARD of the mitochondrial membrane protein IPS-1 (also called Mitochondrial Antiviral Signaling Protein, Virus Induced Signaling Adaptor, or Cardif), which is the common adaptor to all RLR [23–26]. IPS-1 becomes a scaffold for a complex of proteins that recruit and activate TANK-binding kinase (TBK-1) and (in immune cells) IKK-i [27–29]. These kinases phosphorylate and activate the transcription factors IFN regulatory factor (IRF)-3 and IRF-7, which traffic to the nucleus to bind to promoters of IFN production.

Some elements of this pathway, most notably IRF7 and the RLRs, are themselves IFN-inducible, creating a positive feedback loop [30]. Early phases of RLR activation may be enhanced by non-RLR RNA helicases such as DEAD-box helicase 3 (DDX3), which also binds IPS-1 [31]. The involvement of DDX3 and other accessory proteins has not been explored in RABV infection specifically. Emerging studies of other viruses show that regulatory proteins of the RLR pathway are frequently the targets for inhibition by viral proteins ([32, 33]; reviewed in [34]).

The products of this signaling cascade, type I IFN proteins, signal through a separate Janus-activated kinase (JAK)-STAT-dependent pathway (reviewed in [35]). Type I IFNs are exclusively detected by IFNAR, which is expressed on the surface of most mammalian cell types [36]. IFN binding causes IFNAR to oligomerize, triggering phosphorylation of the receptor by JAKs. The phosphorylated receptor becomes a docking site for Signal Transducer and Activator of Transcription (STAT)-1 and STAT2, which are phosphorylated by JAK. The subsequent dimerization between STAT1 and STAT2 creates a novel nuclear localization signal, which binds the transcription factor IRF-9 and translocates to the

nucleus. This complex interacts with the ISRE, which is the promoter element of ISGs, completing the type I IFN signaling pathway.

RABV-P-Dependent Subversion of IFN Signaling

The RABV phosphoprotein P, initially known as the cofactor for RABV polymerase, has emerged as a multifunctional protein that inhibits IFN signaling at the levels of IFN induction and IFN response. P is expressed as a full-length protein as well as in four truncated forms, generated by ribosome initiation at internal AUG initiation codons. Full-length P is the most abundant protein product, but the truncated isoforms play critical roles in subverting antiviral immunity.

The influence of P on the interferon response was discovered when a recombinant RABV (rRABV) expressing a GFP-P fusion protein in place of wild-type P was found to replicate only in cells lacking an IFN response [37]. A yeast two-hybrid screen revealed a direct interaction between STAT1 and P [38]. The interaction mapped to two specific residues on P, Y689, and Y701, which are responsible for STAT1/2 dimerization and nuclear translocation, respectively [39]. In this same study, exogenous expression of P alone was sufficient to prevent nuclear STAT1/2 accumulation in response to IFN. P and STAT1/2 coimmunoprecipitated in extracts of IFN-treated cells but not IFN-untreated cells, indicating that P could be considered an IFN-dependent IFN antagonist. Both nuclear and cytoplasmic forms of P were involved in this process. A nuclear localization sequence on P is required for this activity, though the retention of STAT1/2 in the cytoplasm by cytoplasmic truncated P isoforms also plays a role [40].

In an independent line of research, it was observed that rRABVs engineered to express reduced levels of P induced greatly elevated levels of IRF3 phosphorylation, dimerization, and nuclear import [41, 42]. This change was not abolished by the deletion of the STAT binding regions of P. Alanine-scanning mutagenesis of P revealed a separate mechanism of IFN signaling inhibition, where a small internal domain (amino acids 176–186) drives the inhibition of IRF3 activation [43]. Therefore, P can be said to inhibit both IFN production *and* responsivity.

The capacity of P to inhibit the IFN system has functional consequences in vivo. In the chimeric rRABV CE(NiP), the P protein of the nonlethal Ni-CE vaccine strain of RABV is replaced with that of the challenge strain Nishigahara. This significantly increases pathogenicity in mice after intramuscular inoculation [44], which can be completely abolished by making a further mutation to the STAT binding site of P [45]. Much smaller elevations in pathogenicity were discovered for the corresponding replacement of N and G, and none at all for M and L, emphasizing the impact of P in determining outcomes of disease [46].

Follow-up studies have elaborated the in vivo role of truncated P isoforms. In one experiment, the internal start codons of the Nishigahara P protein in CE(NiP) were eliminated, resulting in a chimera expressing full-length P exclusively [47]. These mutations decreased pathogenicity after intramuscular but not intracranial inoculation, suggesting a specific role for P in determining RABV neuroinvasiveness. One

possible explanation for this phenomenon builds on existing evidence that P interacts with the dynein microtubule (MT) network, either directly [48] or indirectly [49]. The functional importance of RABV–MT interaction and neuroinvasiveness is the subject of ongoing research: In an rRABV expressing a single P isoform (P3), one mutation was sufficient to inhibit both virus–MT interaction and IFN antagonism [50].

The Role of Individual ISGs

Type 1 IFNs induce expression of hundreds of genes [30]. Since diverse viruses activate the same pathway of IFN expression and response, it cannot be assumed that individual ISGs are important to individual viral infections, however, highly upregulated they may be after infection. Of the few ISGs that are well studied, only a handful have demonstrated clear evidence of being restriction factors during RABV infections. Although the 2′-5′OAS and RNase L system have a dramatic impact on many virus infections, it does not appear to affect rhabdovirus replication [51]. Another ISG family, the myxomavirus GTPases (Mx1 and Mx2), restricts VSV [52] but inhibited RABV replication only when expressed as a bovine protein in human cells, not as a human protein [53]. Protein kinase R (PKR), which is also a restriction factor for VSV [54], was dispensable for a normal IFN response to RABV in MEFs, as were RNase L and Mx1 in the same study [55]. Of those commonly studied ISGs that remain, there is compelling evidence of anti-RABV restriction for the PML protein, and for one member of the Ifit family, Ifit2.

PML is primarily expressed in the nucleus, where it forms the scaffold of PML nuclear bodies (PML-NBs). The IFN response is one of several other cellular stressors that trigger the formation of PML-NBs, which also include DNA damage and oxidative stress (reviewed in [56]). There is a direct interaction between PML and RABV P: upon RABV infection, PML binds to full-length P and the P3 isoform containing a nuclear import signal but missing an export signal, becoming sequestered in the nucleus [57]. In the same study, MEFs derived from PML knockout mice grew RABV to impressive 20-fold higher titers. Reconstitution of one specific PML isoform, PMLIV, was sufficient to suppress RABV growth to near wild-type levels [55]. The precise mechanism of PMLIV-mediated RABV restriction is not known, but some aspects of PML biology may offer clues. For example, PML is essential to an apoptotic pathway triggered by type I IFN [58]. Given that the relative virulence of RABV strains correlates inversely with apoptosis [59, 60], this in itself could be considered a mechanism to resist RABV infections.

Ifit2 (or ISG54) is a member of the Interferon-induced with tetratricopeptide repeats (Ifit) family of proteins, named for the protein–protein interaction domains that dominate their structure (reviewed in [61]). Ifit proteins are localized in the cytoplasm and have no known enzymatic function [62]. The best-characterized Ifit protein is Ifit1 (or ISG56), which restricts the replication of a number of positive-stranded RNA viruses in vitro [63, 64], but not most negative-stranded viruses tested [65, 66]. Whereas only Ifit1 has a well-defined RNA binding partner—5′-methylguanosine capped but

2′O-unmethylated RNA ("CAP0") [63, 67, 68]—Ifit2 has the greatest impact on RABV pathogenicity in vivo [3]. This is probably due to the lack of CAP0 during RABV infection, as RABV uses an alternative RNA maturation pathway that does not include the Ifit1 ligand as an intermediate [20]. Interactions between RNA and Ifit2 have also been discovered, *though the relationship between these interactions and the virus life cycle is not well understood.* Although purified human Ifit2 does not share Ifit1's interaction with CAP0-RNA [67], it slows the mobility of short (12–16 nt) poly (A:U) RNAs with or without 5′ triphosphates [69]. The mobility shift can be abolished by mutation of specific amino acid residues suggested by an Ifit2 crystal structure presented in the same study. However, the connection between the RNAs used in this study and viral pathogenicity is controversial because although these poly(A:U) RNAs form dsRNA duplexes (implying that Ifit2 may bind to dsRNA), their short length (<30 nt) makes them likely to be indistinguishable from endogenous siRNAs and microRNAs [70]. Further complicating validation of Ifit–RNA interactions is the IFN-triggered homo- and heterodimerization of Ifit proteins into domain-swapped complexes, which are more likely to be the functional unit of RNA interaction than the individual monomer [62]. The antiviral mechanism of Ifit proteins is expected to be a steric hindrance of viral mRNA translation, based on the fact that Ifit1 [71, 72] and Ifit2 [73] interacts with the eukaryotic translation initiation factor complex (eIF) and block translation in vitro.

IFN-Independent Innate Immunity to RABV

Despite the apparent primacy of the IFN system, several other aspects of innate immunity have an impact on RABV infections. IFNAR knockout mice, whose cells lack any type I IFN sensitivity, still recover from some experimental RABV infections, albeit less frequently [3]. While it is impossible to characterize a phenomenon as truly "interferon independent" unless an IFN-incompetent cell or animal model is specifically used (such as IFNAR knockout mice), studies of RABV and related viruses occasionally describe antiviral mechanisms that do not involve any elements of the IFN pathway or any ISG. These cellular defenses—and the processes by which RABV subverts them—reveal a dynamic relationship between virus and host.

Physical separation of virus components from the cytoplasm may play a role in RABV immune evasion. Cytoplasmic inclusions are known as Negri bodies (NBs), a key diagnostic of RABV in histology, are sites of virus replication and particle assembly [74]. A recent report demonstrated that NBs share certain properties to other liquid organelles such as endosomes: liquid-phase interior, fusion to form larger structures, and spherical shape. This evidence suggests that in addition to facilitating assembly, inclusions such as NBs prevent cytoplasmic pattern recognition receptors from accessing their viral ligands. One possible countermeasure involves cellular stress granules, which were observed adjacent to NBs and accumulate viral mRNAs [75]. In that report, inhibition of stress granule formation increased

viral replication and translation in vitro, an otherwise-unexplored antiviral mechanism.

Furthermore, NBs may recruit cellular proteins that interfere with innate immune signaling. Although a minimal NB-like inclusion can be formed with the expression of N and P proteins exclusively, NBs during infection contain an array of non-virus-derived products [76]. TLR3, an endosomal receptor of dsRNA, is found in NBs and has been demonstrated to be necessary for NB formation [77]. Despite the conventional understanding of TLR3 as a component of the immune system, TLR3 knockout mice had *decreased* pathogenicity following rabies virus infection [78]. The cellular chaperone protein Hsp70 also accumulates in NBs, interacting with RABV nucleoprotein and positively regulating infection [78]. Additionally, many of the other cellular proteins enriched in NBs are polyubiquitinated [78]. This has been taken as support for a hypothesis, originally proposed for DNA viruses [79], that viruses hijack the cellular processes which ordinarily sequester misfolded proteins into chaperone- and ubiquitinated protein-rich aggresomes.

In this proposed model, the cellular scaffold that shields misfolded proteins during autophagy is co-opted by the virus to compartmentalize and protect itself during assembly. The interconnections between autophagy and viral infections are an area of active study for several other negative-stranded RNA viruses, though only a handful of studies have explored rhabdoviruses specifically (reviewed in [80]). The induction of autophagy following VSV infection in vitro was mapped to specific regions of the viral glycoprotein [81], work which has been extended to fish rhabdoviral G proteins in vivo [82]. One attempt to relieve experimental RABV infections with an autophagy-modulating drug has demonstrated promising results [83], though the specific steps of RABV life cycle affected, and the relationship to NBs (if any), remains to be elucidated.

In contrast to NBs, an analog to phase separation is employed by the host to *resist* RABV: the separation between the permissive and adjacent nonpermissive cell, and that of the endothelial blood–brain barrier (BBB). During in vivo RABV infections, the cellular localization of virus growth and host defenses plays a critical role. For instance, evidence has emerged that abortively infected astrocytes can be a source of IFN during experimental intracranial RABV infections, limiting the growth of the virus [84]. This is an effective countermeasure to IFN inhibition by RABV-P, since nonpermissive astrocytes are unlikely to express enough P to counteract ISG induction and amplification of the IFN signal. In this way, the exclusive preference of RABV for neurons during natural (as opposed to intracranial) infections can be seen as a means of avoiding an innate immune response. In a similar sense, the BBB functions as a physiological barrier that can be exploited against RABV. BBB permeability increases during RABV infections, allowing immune effectors such as cytokines and antibodies to enter the CNS [85]. This phenomenon was suggested to partially explain the survival of a human rabies patient in a medically induced coma [86], and has since been elaborated by a number of animal studies [87, 88], though its overall relevance to the survival of that patient remains controversial [89].

Finally, the role of RNA interference (RNAi) in resisting RNA virus infections has been considered in recent years, although RABV has not been studied directly. It

is commonly accepted that the innate antiviral defenses of mammalian and other vertebrate cells are fundamentally distinct from those of plants, fungi, and invertebrates, where the IFN system is absent (reviewed in [90]). In these organisms, cells respond to virus infection by processing viral RNA intermediates into short, ~22 nt virus-derived small interfering RNAs (vsiRNAs) that hybridize to viral RNAs and activate the RNA-induced silencing complex (RISC). In recent years, a controversy has emerged over the question of IFN-sensitive [mammalian] cells retaining antiviral RNAi [91]. In particular, the evolutionary relationships between RIG-I and DLH-1 [92], a coordinator of antiviral RNAi in nematodes, have made this an intriguing hypothesis.

There has been mixed evidence for mammalian antiviral RNAi. Deep-sequencing of RNA from mouse embryonic stem cells (mESCs) infected with encephalomyocarditis virus (EMCV; a picornavirus) revealed apparent vsiRNAs, which were not present in an isogenic Dicer knockout cell line [93]. In the same cells, a strain of Nodamura virus (NoV; a mosquito-borne (+)ssRNA virus) deficient in the nonstructural B2 protein, a previously known suppressor of RNAi, also triggered the production of vsiRNAs and grew poorly. Genetic ablation of the RNAi pathway rescued NoV replication, strongly suggesting antiviral RNAi. In a separate BHK cell model [94], *trans*-complementation with B2 or Ebola virus VP35, which is independently known as a suppressor of RNAi in mammalian cells ([95], had the same effect. Finally, a study of IAV growth in 293T cells has recently found similar results [96].

However, a truly IFN-independent link between mammalian RNAi and viral restriction has not been established. Functional separation of antiviral RNAi and the IFN system is difficult because known inhibitors of vsiRNA production (NoV-B2, Ebola VP35, and others) also interact with the IFN pathway [97]. To the extent observed, mammalian antiviral RNAi has to be "uncovered" by the suppression of elements of the IFN system which strongly inhibit it during infections, such as Lgp2 [98], or by wholesale suppression of IFN signaling [97]. However, potential applications of virus-targeted RNAi have been proposed despite this restriction, such as miRNA targetome mapping of infected cells, or the genetic manipulation of tropism and virulence [97]. To this end, those properties that have made RABV a preferred tool for trans-neuronal tracing—exclusive infection of neurons, trans-synaptic spread, and persistent nonlytic infection [99]—may make RABV a promising context to studying RNAi.

Concluding Remarks

Innate immunity to RABV represents a network of overlapping cellular pathways, centered around but not exclusive to the IFN system. This network, which is the outcome of an ancient arms race, has come to include both elements common to all RNA viruses and elements specific to RABV, or RABV-related lyssaviruses. Given the challenges inherent in treating a viral brain disease, a minute understanding will probably be required to design a therapy. Even those aspects of RABV immunity, which may be considered settled, such as the canonical IFN pathway, or the IFN

inhibitory action of RABV-P, were mostly unknown as little as 20 years ago. This research has numerous applications, especially given the parallel maturation of recombinant DNA technology and computational genomics. RABVs directly expressing immunostimulatory molecules, or precisely impaired at sites of immune subversion, may comprise the next generation of RABV vaccines. The development of novel research tools is also made possible by a better understanding of the host response to RABV, especially in the field of transneuronal tracing. Finally, this ever-increasing understanding renews hope for the development of therapeutic interventions for rabies.

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Novel Rabies Vaccines

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Abstract

Novel rabies vaccines that are less expensive and more immunogenic than current vaccines are needed to reduce the human death toll of rabies. Such vaccines would also allow for more widespread use of rabies vaccines in childhood immunization programs. A number of adjuvants that would allow for dose sparing of current vaccines as well as alternative vaccine prototypes including protein vaccines, genetically modified rabies viruses, pseudotyped viruses, and different types of genetic vaccines are being explored pre-clinically. Some of those have reached early clinical testing. This chapter describes the potential of these different rabies vaccines for use in pre- or post-exposure vaccination.

Introduction

Rabies caused by lyssaviruses, which are divided into three phylogroups and 7 genotypes, claims more than 55,000 human lives annually [1]. Most cases are caused by rabies virus, a phylogroup1 lyssavirus, that forms the basis of current rabies vaccines. About 40% of the deaths occur in children below the age of 15 years. Rabies virus is most commonly transmitted by dogs although other mammals such as cats, raccoons, skunks, foxes, wolves, bats, and others can transmit the virus. In North America, exposure to rabid bats is the main cause for human rabies. Mandatory immunization of dogs has dramatically reduced the incidence of rabies throughout the Americas and Europe but has not yet been successful in Asia and Africa where free-ranging dogs are common. Oral rabies vaccination of wildlife

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H. C. J. Ertl (ed.), *Rabies and Rabies Vaccines*,
https://doi.org/10.1007/978-3-030-21084-7_9

has reduced human rabies virus exposures in Western and Central Europe but is more challenging in the less densely populated areas of the Americas and elsewhere.

Safe and efficacious rabies vaccines for humans are available. They are based on rabies virus that is grown in tissue culture or embryonated eggs and then inactivated. As incubation times for rabies are relatively long and exposures are noteworthy, as they are linked primarily to bites from a rabid animal, vaccines are most commonly given after exposure. Rabies vaccines are given prophylactically to humans at high risk such as veterinarians, wildlife researchers, cavers, or individuals working with the virus. Recently, Peru opted to include rabies pre-exposure prophylaxis (PrEP) into childhood immunization programs in highly endemic remote areas to reduce the increasing death rates due to exposure of children to rabid vampire bats [2, 3]. Recently, the Philippines implemented a similar national program [3, 4].

Currently, licensed rabies vaccines are relatively poorly immunogenic and are thus given repeatedly to achieve adequate titers of neutralizing antibodies, the correlate of protection against infection. For PrEP, rabies vaccines are given three times to reliably induce virus-neutralizing antibody titers $\geq$0.5 international units (IU). Although rabies vaccines induce long-lasting memory B cell responses [5–7], antibody titers eventually wane, necessitating periodic booster immunizations. For post-exposure prophylaxis (PEP), rabies vaccines are given 3–4 times starting as soon as possible after exposure once the wound has been thoroughly cleaned. In case of severe exposure, defined as exposures through transdermal bites or scratches and mucosal contact with saliva or contact with bats, the vaccine is combined with rabies immune globulin (RIG), which should be infiltrated directly to the site of the bite. Rabies vaccines and RIG are expansive and therefore underutilized, which together with lingering ignorance on modes of rabies virus transmission and limited access to health care, results in the high rabies-related human death rates.

More immunogenic vaccines, that achieve virus-neutralizing antibody titers after a single dose and that are less costly, would be expected to increase public access to PrEP and PEP and thereby reduce human death due to rabies. Currently, cost can be reduced by using ID rather than IM immunization. Vaccine injected ID has ready access to the rich network of dendritic cells within the skin [8], which are scarcer within muscle tissue. ID immunization, therefore, triggers more potent immune responses and allows for a reduction of the vaccine dose. Current rabies vaccines given ID at a 5- to 10-fold lower dose compared to the IM dose induce adequate antibody titers. Another way to reduce the cost for rabies vaccinations and increase compliance with recommended regimens is to reduce the number of vaccine doses and tighten immunization schedules from several weeks to one week (https://www.who.int/rabies/PEP_Prophylaxis_guideline_15_12_2014.pdf).

Thus far these changes in rabies vaccination, which are now recommended by WHO and which already prior to that were used extensively in many rabies-endemic countries, have not reduced the efficacy of the rabies vaccine.

This chapter discusses the pros and cons of novel human rabies vaccines under development.

Vaccine-Induced Immune Correlates of Protections

Protection against infection or spread of rabies virus is mediated by neutralizing antibodies that cross-react among viral species within lyssavirus phylogroup 1 [9, 10]. Antibodies induced by current vaccines fail to neutralize phylogroup 2 and other genetically disparate lyssaviruses [11, 12]. Neutralizing antibodies are directed against the lyssavirus glycoprotein, the only protein expressed on the surface of the bullet-shaped virions. The viral glycoprotein forms trimers and most of the neutralizing antibodies are directed against conformation-dependent epitopes. Thus, rabies vaccines must express the viral glycoprotein in its native conformation to induce neutralizing antibodies [11]. Memory B cell responses to current rabies vaccines are long lived and can be recalled decades after the initial immunization [13]. Vaccinated individuals that are exposed to rabies and related lyssaviruses therefore only require two booster immunizations without RIG rather than the full PEP regimen. Induction of B cell responses to the rabies vaccine requires help from $CD4^+$ T cells [14]. Vaccination of individuals with T cell immunodeficiency such as patients suffering from AIDS may thus not result in adequate antibody titers [15]. Most of the antigens of rabies virus, including the glycoprotein, carry T helper cell epitopes [16]. Induction of $CD4^+$ T cells and B cells necessitates stimulation of an initial innate response to drive activation of antigen-presenting cells. Currently licensed rabies vaccines do not contain an adjuvant and rely on intrinsic viral factors to induce inflammatory reactions, such as the single-stranded RNA genome that activates Toll-like receptors 7/8 [17] and double-stranded loops that can activate RIG-I helicase [18].

Requirements for Next-Generation Rabies Vaccines

Novel rabies vaccines need to equal current vaccines in safety and efficacy and surpass their immunogenicity to allow for single-dose regimens and an overall cost reduction. They need to induce innate immunity, which can be achieved with adjuvants. Adjuvants affect the flavor of immune responses by driving activation of type 1 or 2 T helper (Th) cells. Both promote stimulation and affinity maturation of B cells but they achieve different types of class switching. Th1 cells in humans promote stimulation of IgG1 and IgG3 while Th2 responses favor switching to IgG4 and IgA. Although it is currently unknown which antibody isotype is best suited to protect humans against rabies, some evidence obtained in mice suggests that antibodies with Th1-linked isotypes can more readily be induced within the CNS, where they may promote virus clearance [19]. Novel vaccines must express the rabies virus glycoprotein in its native form and they need to induce an antibody response that broadly neutralizes all phylogroup 1 lyssaviruses. Neutralization of phylogroup 2 lyssaviruses or other recently isolated lyssaviruses that belong to neither phylogroups 1 nor 2 [20] would be desirable. Vaccines should induce sustained antibody and memory B cell responses. Vaccines used for PEP must induce an antibody response rapidly before the virus spreads into the nervous

system. As less costly rabies vaccines are most direly needed in developing countries, the vaccine must be heat-stable as cold chains are expensive and difficult to maintain. Delivery needs to be easy.

Several vaccines and vaccine adjuvants have undergone pre-clinical and in part clinical testing. Some meet the criteria required for vaccines use for PEP and PrEP, while others are only suited for PrEP.

Adjuvants

Adjuvants increase the immunogenicity and potency of vaccines by stabilizing the antigen and/or affecting its prolonged release, targeting specific cells or promoting inflammatory responses. Adjuvants that have the latter effect by their very nature increase the reactogenicity of vaccines and on rare occasions even result in serious adverse events as was shown with adjuvanted influenza vaccines [21]. Although tremendous research efforts have focused on the development of new adjuvants very few have reached licensure. Aluminum salts in the form of aluminum hydroxide, aluminum phosphate, alum (potassium aluminum sulfate), or mixed aluminum salts have been approved in the United States for more than six decades. Two other adjuvants, i.e., AS04 a combination of aluminum hydroxide and monophosphoryl lipid A, are approved for use with Cervarix, a virus-like particle (VLP) vaccine against oncogenic types of human papillomaviruses [22] and AS03, an oil-in-water emulsion, for H5N1 that is currently not used in the United States in humans but is being stockpiled in case of an avian influenza outbreak [23]. AS03 has been used outside the United States in 47 countries together with the pandemic H1N1 vaccine, where it was linked to increases in narcolepsy in children [24]. No other adjuvants have been licensed in the United States thus far, although many have undergone pre-clinical and early clinical testing. Adjuvants that have been tested in animals with inactivated rabies vaccines are listed in Table 1. Most were tested in mice, where they increased antibody responses to the rabies vaccine and thus protection against challenge. The caveat should be pointed out that results obtained in rodents, especially with adjuvants that target pathogen recognition receptors [37], may not necessarily translate to humans due to differences in innate receptor specificity and distribution [38].

Data obtained in humans and nonhuman primates indicate that an alum-adjuvanted rabies vaccine would provide limited advantages [25]. Two adjuvants, ISCOMATRIX™, a particle forming adjuvant composed of cholesterol, phospholipid, and saponin and IMO-2170, an immune modulatory oligonucleotide with agonist activity to TLR-9, yielded promising results in nonhuman primates [30]. One adjuvant, a TLR-3 agonist based on a synthetic dsRNA analogue and a refined form of polyinosinic-polycytidylic acid stabilized with kanamycin and calcium has been tested in human volunteers with no prior history of rabies exposure or vaccination [36]. Group 1 received RABIPUR in a 4-dose i.m. regimen (1-1-1-1), group 2 received the same regimen with the adjuvanted vaccine called PIKA® rabies vaccine, group 3 received the PIKA® rabies vaccine in an accelerated 3 visit regimen

Table 1 Adjuvants tested with conventional rabies vaccines

Adjuvant	Vaccine	Species	Study outcome	References
Aluminum hydroxide	HDCSV + adjuvant given s.c. compared to HDCSV, i.m., single or 8-site i.d. regimen	Humans	Response to vaccine with adjuvant equal to single-site i.d. regimen without adjuvant, inferior to 8-site i.d. regimen	[25]
IL-2, given daily systemically	Attenuated SAD strain of rabies	Outbred mice	Enhanced vaccine potency tested for by challenge with CVS-11 virus	[26]
Polar glycopeptidolipids from *Mycobacterium chelonae*	Semple vaccine	BALB/c mice	Enhanced vaccine potency tested for by challenge with CVS virus	[27]
CpG ODN (BW006)	Inactivated rabies virus strain CTN	BALB/c mice	Increased titer of neutralizing antibodies, increased protection upon challenge	[28]
Activation-associated protein-1 from *Onchocerca volvulus*	VERORAB	BALB/c mice	Increased IgG1 and IgG2a antibody responses to the rabies vaccine	[29]
ISCOMATRIX™ adjuvant	Rabavert®	Rhesus macaques	Significantly increased titers of neutralizing antibodies	[30]
IMO-2170, synthetic TLR9 agonist	Rabavert®	Rhesus macaques	Significantly increased titers of neutralizing antibodies	
Amorphous aluminum hydroxylphosphate sulfate	Rabavert®	Rhesus macaques	Marginally increased titers of neutralizing antibodies	
Uridine 5′-triphosphate	Commercial rabies vaccine (single dose)	BALB/c mice	Marginally increased protection in PrEP compared to a suboptimal dose of vaccine	[31]
Ginsenoside Re (Re) is a saponin from Panax ginseng	Rabvac®	Outbred mice	Enhanced and prolonged antibody responses	[32]
Salmonella typhimurium flagellin	Whole-killed rabies vaccines	BALB/c mice	Slightly increased antibody responses	[33]

(continued)

Table 1 (continued)

Adjuvant	Vaccine	Species	Study outcome	References
Polysaccharides IIP-A-1 and IIP-2 from *Isatis indigotica* root	Whole-killed rabies vaccines	BALB/c mice	Accelerated and increased antibody responses	[34]
Hydrogenated soya phosphatide and cholesterol liposomes	Whole-killed rabies vaccines	BALB/c mice	Slightly increased vaccine potency	[35]
TLR-3 agonist	RABIPUR®	Humans	Well tolerated, increased immunogenicity of the vaccine	[36]

(2-2-1). The experimental vaccine was well-tolerated although 1/12 subjects of group 2 had to be taken off the trial due to pruritus that eventually resolved. Group 3 developed titers of rabies neutralizing antibodies faster than control group 1 and antibody responses were more sustained. Group 2 showed a trend toward accelerated and prolonged responses, which failed to reach significance. Although the results of phase I safety trial were promising, questions on improved immunogenicity remain. They will need to be addressed in a larger phase II trial. Specifically, the most promising accelerated 2-2-1 regimen for the PIKA® rabies vaccine was compared to the standard 1-1-1-1 regimen, which opens the question if the faster and enhanced response was indeed caused by the adjuvant or differences in dosing and timing of vaccination.

Protein and Peptide Vaccines

Protein or peptide vaccines are based on the viral glycoprotein, a 65-k Da protein that contains an intracytoplasmic domain, a hydrophobic transmembrane domain, and an ectodomain. After synthesis, the protein forms trimers and is N-glycosylated minimally at one of three potential sites, before it is transported to the cell surface [24]. Protein vaccines based on the rabies virus glycoprotein have the advantage that they are exceptionally safe and they can be used for both PEP and PrEP. Depending on the source of the protein, such as plant cells, they may also be cost effective. Disadvantages of protein vaccines are that correct folding into the native trimeric structure of the rabies virus glycoprotein, which is essential for the induction of neutralizing antibodies, remains a challenge. Full-length glycoprotein is poorly soluble due to the high hydrophobicity of the transmembrane domain [39], which is nevertheless essential for the protein's correct folding.

Different types of protein and peptide vaccines that have been explored are listed in Table 2. Rabies virus glycoprotein produced in mammalian cells, such human embryonic kidney (HEK) 293 cells [46], baby hamster kidney (BHK)-21 cells [40],

Table 2 Protein and peptide vaccines to rabies virus

Expression system	Protein	Immunization protocol	Species	Results	References
Protein vaccines					
Mammalian expression systems					
HEK-293 cells	VLP-forming glycoprotein	Two immunizations	Mice	Protection	[40]
BH-K21 cells	Glycoprotein	No immunization studies			[41]
NA cells	Glycoprotein	No immunization studies			[41]
Insect cell expression systems					
Baculovirus in Sf9 cells	Purified glycoprotein + alum	2 doses, 200–400 ng	Mice	Protection	[36]
	Unpurified glycoprotein	~12–120 μg	Mice, Racoons	Protection	[30, 42]
	Glycoprotein nanoparticles	1–5 μg	Mice	Superior immunogenicity to Rabipur	[43]
		2–3 doses, 5–50 mg	Humans	Results unknown	[44, 45]
Drosophila melanogaster Schneider 2 cells	Glycoprotein	No immunization studies			[46]
	Purified glycoprotein	3 μg of purified protein	Mice	Modest neutralizing antibody response	[39]
Yeast expression systems					
Saccharomyces cerevisiae	Unpurified glycoprotein	Yeast extract	Mice	No protection	[40]
Pichia pastoris	Glycoprotein	No immunization studies			[41]
Pichia angusta	Truncated glycoprotein	No immunization studies			[47]
Plant expression systems					
Maize	Unpurified glycoprotein	1 dose, 50 μg, orally	Mice	Protection	[48]
		1 dose, 0.5–2 mg, orally	Sheep	Partial protection	[49]
Tobacco plants	Purified glycoprotein + Freund's adjuvant	4 doses, 25 μg, i.p.	Mice	Protection	[50]

(continued)

Table 2 (continued)

Expression system	Protein	Immunization protocol	Species	Results	References
Spinach	Tobacco mosaic virus expressing antigenic determinants of rabies virus	3 doses, 50 μg, i.p.	Outbred mice	Partial protection	[51]
		3 doses, spinach leaves, orally	Humans	Induction of primary and recall B cell responses in some individuals	[50, 52]
Carrots	Rabies virus protein in raw carrots	50 μg of glycoprotein + 50 μg of nucleoprotein	Mice	Partial protection	[53]
Peptide vaccines					
Branched lipopeptide vaccine	$CD4^+$ and $CD8^+$ T cell epitopes	3 doses, 50 μg, i.p.	Mice	Induction of T cells	[54]
Lipopeptide vaccine adjuvanted with TLR-7	$CD4^+$ and $CD8^+$ T cell epitopes + TLR-7 agonist	1 dose, 50 μg combined with a rabies vaccine	Mice	Partial protection	[55]
Multi-epitope-based vaccine coated with canine gp69	conserved B cell epitopes coated with canine gp69 and given in an oil in water emulation	1 dose	Mice, dogs	Partial protection	[56]

or neuroblastoma cells [41] is glycosylated, although the glycosylation pattern, which in turn affects the protein's immunogenicity, varies depending on the host cell type. It is furthermore affected by culture conditions. Expression in insect cell systems, such as by baculovirus in *Spodoptera frugiperda* (Sf-9) cells [47, 48] or in *Drosophila melanogaster* Schneider 2 cells [50, 53], also results in oligomeric glycoprotein able to induce virus-neutralizing antibodies in mice. A baculovirus-derived glycoprotein that spontaneously forms micelles (nanoparticles) has undergone testing in mice. A 3-dose regimen induces higher rates of seroconversion than commercial Rabipur vaccine. This vaccine, developed by CBL Biological, has undergone phase I and II trials in humans and a phase III trial is planned. Results of these trials are not yet available to the public.

Expression in *Saccharomyces cerevisiae* [51] resulted in a protein that failed to be immunogenic in mice, which may relate to the typically high mannose glycosylation pattern by yeast. Expression in methylotrophic yeast *Pichia pastoris* was also assessed, resulting in a protein that at least in part was correctly folded [52]. Similar results were obtained in *Pichia angusta* (*Hansenula polymorpha*) [54]. Neither protein was assessed for induction of neutralizing antibodies in animals, the ultimate test for correct folding and glycosylation of the rabies virus glycoprotein.

The rabies virus glycoprotein has been produced in plant cells, including maize [56], *Nicotiana tabacum* plants [57], carrots [58], or spinach [59, 60]. The latter expressed epitopes of the glycoprotein and the nucleoprotein together with the alpha mosaic virus coat protein to facilitate oral vaccine delivery. Plant cells appropriately glycosylate the rabies virus glycoprotein. However, successes upon oral immunization varied. A maize-derived glycoprotein fed at 50 μg/dose within the kernels to mice resulted in protection against challenge with a vampire bat virus [56]. The chimeric spinach-derived peptide vaccine was initially tested after intraperitoneal injection of mice, where it achieved protective immunity after three doses [59]. Humans were fed three times raw spinach containing the chimeric rabies virus peptides [60]. Volunteers without prior immunization with the rabies vaccines were then immunized with one dose of a commercial rabies vaccine. Seven days, later 3 of 9 individuals had developed neutralizing antibodies, indicating that the plant vaccine had primed a B cell response in at least some of the human volunteers. In another arm of the study, humans who had previously been immunized with a commercial rabies vaccine were fed the same material. In this group, 3 of 5 showed a recall response. Another study demonstrated in mice the immunogenicity and efficacy of a purified rabies virus glycoprotein grown in *Nicotiana tabacum* leaves [57] or in carrots [58].

Results obtained with some of the expression systems, especially the virus-like particles (VLPs) formed in HEK 293 cells and the maize-produced glycoprotein, are promising. Unless the glycoprotein is used for oral immunization, it must be purified, which adds another layer of complexity and cost to protein vaccines. Oral immunization with raw material, such as maize kernels, carrots, or spinach leaves, may provide challenges of accurate dosing, not only of the ingested material but also of the amount of antigen that eventually is presented to the immune system. Several groups have explored the use of peptides. A branched lipopeptide vaccine, with or

without TLR7 agonist, was shown to induce T cell responses to rabies virus that could accelerate the B cell response to a traditional vaccine [61]. A multi-epitope-based vaccine coated with canine gp69 tested in mice showed limited efficacy [62]. Considering that rabies vaccines need to induce a very broad antibody response against multiple isolates and preferentially different genotypes, this approach is unlikely to replace current vaccines.

Genetically Altered Rabies Vaccines

Rabies virus can be modified by reverse genetics [63]. This has led to the production of highly attenuated rabies virus and/or virus with improved immunogenicity that could be used for animal or human vaccination (Table 3). Depletion of the P gene, which encodes part of the viral polymerase, attenuates rabies virus. The virus is apathogenic even if injected intracerebrally into adult or suckling immunocompetent mice or into immunodeficient mice, although in the latter the P gene-deleted vaccine spread from the periphery into the central nervous system. The P gene-deleted rabies virus induces a virus-neutralizing antibody response [64]. The response comes up fairly slowing but then surpasses that of an inactivated vaccine based on wild-type virus, suggesting that such a construct may be considered for PReP, but not PEP. To enhance the immunogenicity of the P-gene deleted rabies vaccine, it was further modified to express two copies of the viral glycoprotein gene. This vaccine more rapidly induced rabies virus neutralizing antibodies in mice and nonhuman primates [65]. Rabies virus depleted of the matrix (M) gene is also apathogenic and unlike the P gene-deleted virus does not disseminate into the brain of immunodeficient mice. This vaccine very rapidly induces virus-neutralizing antibodies in mice and a somewhat slower response in nonhuman primates [66]. Both types of viruses induce a Th1-biased response.

Attenuated viral vaccines are attractive, as they produce higher and more sustained levels of antigen than an equal dose of an inactivated virus. Nevertheless, considering that rabies is nearly always fatal, a live vaccine, even if it is shown by all possible means to be safe in animals, may not be accepted by regulatory authorities or the public. Attenuated rabies vaccines could be useful for the treatment of human patients with active symptoms of a CNS rabies virus infection [67].

To overcome this potential safety limitation, mutants, which carry two copies of the glycoprotein gene, were inactivated and then tested in dogs, in comparison to a traditional vaccine [68]. After a single dose, the mutant virus-induced an accelerate antibody response that protected ~80% of the animals against a challenge with a virulent strain of rabies virus. This vaccine could be suitable for PEP where the speed of onset of a virus-neutralizing antibody response is of the essence. A further advantage of the mutant vaccine is its superior growth in BHK-21 cells, compared to the parental HEP-Flury strain, although additional studies are needed to assess if this endures scale-up of vaccine production.

Table 3 Genetically altered rabies vaccines

Rabies virus	Type of vaccine	Immunization protocol	Species	Results	References
P gene-deficient rabies virus	Live attenuated virus	1 dose, 10^6 FFUs, i.p.	Mice	Induction of neutralizing antibodies and protection against challenge, superior to the efficacy of an inactivated wild-type rabies virus	[58]
Modified P gene-deleted rabies virus	Live attenuated virus	1 dose,10^3–10^5 FFUs, i.m.	Mice	Rapid induction of neutralizing antibodies and protection against challenge	[59]
M gene-deficient rabies virus	Live attenuated virus	1 dose, 10^3–10^5 FFU, i.m.	Mice	Rapid induction of neutralizing antibodies, complete protection against challenges	[60]
		2 doses, 6×10^7 FFUs, i.m.	Nonhuman primates	Induction of neutralizing antibodies	
Rabies virus with two glycoprotein gene copies	Inactivated virus	1 dose, equivalent of 10^7 FFU with adjuvant, s.c.	Dogs	Slightly accelerated neutralizing antibody response, 83% protection against challenge	[62]

FFU focus forming unit

Virus Particle Vaccines

Some viruses can be modified to express the rabies virus glycoprotein on the surface of the virion (Table 4). These viruses share the advantage of protein vaccines or genetically modified rabies virus in that the protein is instantly available for induction of immune responses thus potentially allowing for their use in PEP. They furthermore have the advantage that the inflammatory response to the parent virus renders them independent from added adjuvants. Purification methods for viruses are

Table 4 Pseudotyped viruses as vaccines to rabies virus

Pseudotyped virus	Immunization protocol	Species	Results	References
New castle disease virus	1 dose, ~10^6–10^8 egg infective doses, i.m.	Mice	Complete protection	[63]
	3 doses, ~10^8–10^{10} egg infective doses, i.m.	Cats, Dogs		
Baculovirus	2 doses, 10^8 IFU, i.m.	Mice	Complete protection	[64]
Parainfluenza virus 5	1 dose,10^8 PFU, i.m., i.n., oral, PrEP	Mice	Complete protection	[65]
	3 doses, 10^7 PFU, intracerebrally, PEP	Mice	Partial protection	[69]

EID egg infectious unit, IFU infectious unit, PFU plaque-forming unit

well established and not affected by peculiarities of the rabies virus glycoprotein such as the high hydrophobicity of its transmembrane domain. Potential disadvantages are pre-existing immunity to the parent virus or modest levels of the displayed rabies virus glycoprotein, both of which could dampen immune responses to rabies virus. One also needs to consider an increased potential for adverse events due to pathogenicity of the parent virus.

Newcastle disease virus, an avian parainfluenza virus, was modified to express the rabies virus glycoprotein on its surface. The vaccine could be grown to high titers in embryonated chicken eggs and was shown to be safe in mice, dogs, and cats. It induces high and sustained titers of rabies virus-neutralizing antibodies. A single intramuscular dose achieved complete protection in mice, while a triple immunization regimen was shown to protect cats and dogs [70].

A baculovirus was modified to express the rabies virus glycoprotein on its surface and to simultaneously express another glycoprotein under the control of the CMV promoter. This vector thus serves as both a VLP and a genetic vaccine. In mice two, doses of the recombinant baculovirus induce a virus-neutralizing antibody response and complete protection against challenge [71].

Parainfluenza virus 5 vectors expressing the rabies virus glycoprotein were developed and tested initially in a PrEP regimen, where they induced neutralizing antibodies in mice and complete protection against challenge [72]. In the same study, the pseudotyped virus was tested in a PEP regimen in mice. Mice were challenged intramuscularly with rabies virus and then vaccinated three times intracerebrally with the recombinant virus starting 4–6 days after challenge. A significant reduction in clinical signs was observed, although it should be noted that early vaccination with the wild-type virus also reduced illness [72].

Although the pseudotyped virus may be a cost-effective alternative for PEP, additional studies are needed in more relevant animal models.

Genetic Vaccines

Genetic vaccines are vaccines that introduce genetic material or transcripts of a pathogen. Such vaccines need to transduce cells. They use the host cell machinery to produce the immunogen, which then stimulates an immune response. Onset of an immune response to a genetic vaccine is delayed and although this delay may be marginal and synthesis of proteins would be expected to occur within hours leading to steady accumulation of antigen over a span of several days, it nevertheless precludes the use of genetic vaccines for PEP, where speed of the neutralizing antibody response determines whether a patient will live or die.

Genetic vaccines that have been explored for vaccination against rabies virus can be subdivided into mRNA vaccines, DNA vaccines in the form of plasmid vectors or replicons, viral, and bacterial recombinant vaccines (Table 5).

mRNA Vaccines

Pre-clinical studies showed that mRNA encoding the rabies virus glycoprotein injected into animal transduces cells and stimulates a Th2-biased antibody response against rabies virus that protects mice and pigs against challenge [103]. Based on these promising results, a lyophilized mRNA vaccine encoding the glycoprotein of the Pasteur strain of rabies virus, termed CV7201, was tested in a dose escalation trial in human volunteers without previous exposure to a rabies vaccine [104]. The vaccine was given 2 or 3 times intramuscularly on days 0, 28, and 56 or 3 times intradermally on days 0, 7, and 28. Injections were either given by a syringe or an injector device. Ninety percent of participants reported side effects, in 12% of those side effects were considered severe. Local side effects were more common after intradermal application, systemic side effects tended to increase at higher vaccine doses. Intramuscular or intradermal immunization by syringe failed to elicit rabies virus-specific neutralizing antibody titers at or above 0.5 IU/ml. Using injector devises about ~50% of individuals that received the vaccine at the highest dose (400 μg/dose) intramuscularly and ~70% of those injected intradermally developed titers of or above 0.5 IU/ml. By one year after immunization titers in all individuals declined to below 0.5 IU/ml. Some of the individuals who had been primed intradermally with the injector device (80 μg/dose, 3 doses) were boosted one year later with the same vaccine dose, route, and type of injection used for priming. After the boost, ~60% of individuals achieved antibody titers above 0.5 IU/ml. Although this proof of principle studies shows that an mRNA vaccine can induce an immune response in humans, the trial outcome is far from impressive. Considering the severity of rabies, a vaccine that does not achieve adequate titers in all vaccine recipients is not acceptable.

Table 5 Genetic vaccines

Type of vaccine	Immunization protocol	Species	Results	References
mRNA vaccines				
mRNA, PrEP	2–3 times, i.m. or i.d., syringe or injection devise	Humans	Short-lived titers of neutralizing antibodies in some volunteers	[67]
DNA vaccines				
DNA, PrEP	1–2 doses, 0.1–50 μg, i.m. or i.d., syringe or gene gun	Mice	Protective immunity	[73–75]
DNA encoding chimeric glycoprotein, PrEP	1 dose, 5–80 μg, i.m. after cardiotoxin treatment	Mice	Broadly cross-reactive titers of neutralizing antibodies	[76]
DNA, PrEP	2 doses, 100–300 μg, i.m. or i.d.	Dogs, cats	Neutralizing antibodies, protection against challenge	[77]
DNA, PrEP	1 dose, 100–1000 μg, i.m.; 6–60 μg, gene gun	Nonhuman primates	Sustained neutralizing antibody responses, protection against challenge	[78]
DNA + genetic GM-CSF adjuvant, PrEP	1 dose, 1–5 μg, i.m.	Mice	Increased vaccine efficacy	[79]
DNA + monophosphoryl lipid, PrEP	2 doses, 2 μg, various routes	Mice	Increased antibody responses after priming	[80]
DNA + alum, PrEP	1 dose, 100 μg, i.m.	Mice	Increased antibody responses and protection	[81]
DNA + alum or cationic lipid, PrEP	2 doses, 200 μg, i.m.	Horses	Neutralizing antibody responses to DNA vaccine + adjuvant	[82]
DNA + amine-terminated poly (ether imine) dendrimer, PrEP	3 doses, 90 μg, i.m.	Mice	Increased antibody responses and protection	[83]
DNA + Emulsigen-D, PrEP	3 doses 100 μg, i.m.; 1 μg, gene gun	Mice	Increased antibody responses and protection	[84]
DNA prime, adenovirus vector boost, PrEP	1 dose, 50 μg, various routes	Mice	Increase antibody responses in presence of pre-existing neutralizing antibodies to the viral vector	[85]
DNA, PEP	5 doses, 100 μg, i.m. + RIG	Mice	Partial protection	[86]
	1 dose, 100 μg, i.m.			[87]

DNA + Emulsigen-D, PEP	5 doses, 100 μg, i.m.	Mice	Complete protection	[84]
DNA	1 dose, 60 μg, gene gun + RIG	Nonhuman primates	Partial protection	[88]
DNA, PEP	4 doses, 50–100 μg, i.n.	Mice, rabbits	Partial protection	[89]
Replicon vaccines				
Sinbis replicon	1 dose, 50 μg, i.m.	Mice	Complete protection	[90]
Poxvirus vaccines				
Vaccinia virus	1 dose, various amounts and routes	Numerous species	Complete protection	[91–94]
Modified Vaccinia Ankara	1 dose, 10^7–10^9 pfu, i.m.	Mice	Partial protection	[95]
Canarypox virus	1 dose	Cats and dogs	Protection	[96, 97]
Canarypox virus	1 dose, $10^{3.5}$–$10^{5.5}$ $TCID_{50}$, i.m.	Humans	Induction of modest neutralizing antibody responses	[98]
Parapoxvirus	2 doses, 10^8–10^5 $TCID_{50}$ various routes	Mice	Complete protection at the higher doses	[99]
	2 doses, $10^{7.5}$ $TCID_{50}$, i.m. or s.c	Dogs	Rapid onset of neutralizing antibody responses, and enhanced antibody titers at higher doses	[99]
	2 doses 10^5–10^8 $TCID_{50}$, i.m.	Cats	Neutralizing antibody titers	[99]
Adenovirus vectors				
Replication-competent adenovirus (HAdV5)	1 dose, 10^5–10^8 IU, oral	Mice, skunks	Induction of neutralizing antibodies in most animals	[99]
Replication-defective adenovirus (HAdV5)	1 dose, 2×10^4–2×10^6 IU, various routes	Mice	Complete protection upon i.m. immunization	[100]
Replication-defective adenovirus (SAdV25)	1 dose, 5×10^3–5×10^7 IU, s.c. or i.n.	Mice	Complete protection at the higher doses	[101]
Replication-defective adenovirus (SAdV25)	1 dose, 10^9 vp, i.m.	Nonhuman primates	Complete protection	[102]

DNA Vaccines

Plasmid vectors also called DNA vaccines can be given by intramuscular or intradermal injection. They can be applied intradermally upon coating to gold beads with a gene gun. The plasmids transduce cells locally and then produce the antigen [73]. Transduction rates can be increased by electroporation following vector injection [74]. DNA vaccines have clear advantages. They are very easy to produce. They are heat stable. They carry their own adjuvant in the form of CpG sequences within the vector genome, which activate TLR-9 [75]. Their testing in humans has shown that they are well tolerated [76, 78]. They induce a full range of immune responses including Th1-based antibodies. Immune responses tend to be sustained. Their main disadvantage is that their potent immunogenicity in animals has not reliably translated to human studies [77].

Several studies have tested DNA vaccines expressing the rabies virus glycoprotein. Initial studies showed that a single dose of 50 μg of a DNA vaccine given i.m. protected 50% of mice against challenge while 3 doses achieved 80% protection [79]. Subsequent studies reported complete protection after a single i.m. dose of 10 μg of DNA [80] or 2 μg given by gene gun [81]. Using a DNA vaccine expressing a chimeric glycoprotein composed of phylogroup 1 and 2 lyssaviruses or 2 DNA vaccines expressing the entire glycoprotein sequences of these two viruses resulted in a broadly neutralizing antibody response that neutralized most genotypes of lyssavirus [82]. Other studies showed induction of virus-neutralizing antibody responses in nonhuman primates [83], dogs, and cats [84] that as far as was tested protected against challenge. Responses could be increased by adding either genetic adjuvants in form of a second DNA vector expressing a cytokine [85] or by formulating the DNA vaccine in a traditional adjuvants such as monophosphoryl lipid [87], alum [86], cationic lipids [88], amine-terminated poly(ether imine) dendrimer [89], or Emulsigen-D [90]. Alternatively, responses could be enhanced by using a second vaccine such as an adenovirus vector for a booster immunization [105]. In such prime-boost regimens, DNA vaccines were shown to overcome impairment of transgene product-specific B cell responses by pre-existing neutralizing antibodies to the viral vaccine vector [105]. The initial studies focused on PrEP although a number of studies reported reduced mortality by using rabies DNA vaccine in PEP regimens in mice or nonhuman primates [106–109].

Viral replicons such as those based on Sindbis virus replicons were also shown to induce protective levels of neutralizing antibodies in mice and dogs after a single dose [110].

DNA vaccines for other pathogens, such as *Plasmodium falciparum* [91], HIV-1 [92], Hantaan virus [95], Ebola, and Marburg viruses [96] have undergone clinical testing. Immunogenicity was variable. More potent responses were achieved in clinical trials that used DNA vaccines for priming followed by a boost with a viral vector [98]. Such an approach would not simplify current rabies vaccine regimens.

Viral Vector Vaccines

Recombinant viruses, similar to DNA vaccines, induce immune responses after they infect cells in vivo and transcribe the inserted sequence. They have the advantage over DNA vaccines that infection rates are higher which together with more potent signaling to the innate immune system increases their immunogenicity. Their safety profile varies, those that are replication-defective such as E1-deleted adenovirus (Ad) vectors are generally well tolerated [99, 111, 112] while some of the poxvirus vectors are too reactogenic for use in humans [113]. One clear disadvantage of viral vector vaccines is that their immunogenicity is reduced in the presence of pre-existing vector-specific neutralizing antibodies induced by natural infections or previous vaccinations [114, 115]. They are thus suitable for single vaccine regimens but the same vector should not be used for repeated immunizations. Single-cycle flavivirus vectors expressing the rabies virus glycoprotein have been developed. They showed immunogenicity and efficacy against a rabies virus challenge in experimental animals [116]. This vaccine platform has not yet been tested in clinical trials and it is thus impossible to predict its potential for cost-effective scale-up and its performance in humans.

Poxvirus Vectors

Several types of poxviruses have been vectored and used as rabies vaccines (Table 5). Vaccinia virus recombinants expressing the rabies virus glycoprotein are being used for immunization of wildlife [100–102, 117–119]. Although they are highly immunogenic and reliably induce protective immunity after a single dose, their residual virulence precludes their use in humans [120]. Vectors based on Modified Vaccinia Ankara (MVA) are more attenuated. The virus, upon serial passages in cell lines that caused deletions of ~10% of its genome, is no longer capable to replicate in primate cells. This attenuation reduces the vectors' immunogenicity and in mice, an MVA recombinant expressing the rabies virus glycoprotein achieved only partial protection [119]. Other poxviruses, such as canarypox virus, have been vectored to express the rabies virus glycoprotein. The canarypox vaccine showed efficacy in cats [100] and is now licensed for this species as PureVax feline rabies for 1- and 3-year duration of immunity vaccines. The vaccine was also tested in a three-dose regimen in human volunteers in comparison to a traditional tissue culture-derived rabies vaccine. The vaccine was well-tolerated and induced rabies virus-neutralizing antibodies. Titers, which contracted rapidly, were well below those achieved with the commercial vaccine [101]. A recombinant parapoxvirus induced adequate titers of neutralizing antibodies in mice, dogs, and cats but again its immunogenicity was below that of the vaccinia virus recombinant [102].

Poxvirus vectors, although licensed for wildlife immunization and for routine vaccination of cats are overall poor candidates as single-dose vaccines for humans—those that are highly immunogenic are too reactogenic and those that are more attenuated lack immunogenicity.

Adenovirus Vectors

Adenoviruses cause species-specific infections. Multiple serotypes have been isolated from various species and some of those derived from human or simian serotypes have undergone clinical testing as preventative vaccines for a plethora of pathogens [99, 111, 112, 121–123].

Adenovirus vectors can be constructed to retain their replication competence by inserting foreign sequences into the deleted E3 domain that encodes polypeptides that are non-essential for virus replication but serve to subvert immune responses. Replication-competent adenovirus vectors based on human serotype 5 (HAdV5) expressing the rabies virus glycoprotein have been licensed in North America for immunization of wildlife [124]. Due to their potential virulence in their human host where wild-type HAdV5 virus can cause pneumonia, gastroenteritis, and/or hepatitis, such vectors are not suited for use in humans [125].

Adenoviruses are rendered replication defective by insertion of sequences into the deleted E1 domain, which encodes proteins that are essential for the transcription of the other viral genes. E1-deleted adenovirus vectors induce very potent T and B cell responses that, due to low-level persistence of the viral vectors, are exceptionally sustained [126]. They are well tolerated in humans if used at immunogenic doses. High doses elicit severe side effects due to the induction of strong innate immune responses. Production and purification methods for use in humans are well established [127]. Methods to preserve adenovirus vectors independent of cold chains are available [43, 128]. It is likely that adenovirus vector vaccines for rabies virus would be cost effective, as it is estimated that a single-dose vaccine could cost as little as one dollar [44].

The main disadvantage of adenovirus vectors is that their immunogenicity and efficacy are impaired by pre-existing neutralizing antibodies to the vector [114, 115]. Adenoviruses are ubiquitous and most humans become infected early during childhood with different serotypes. The prevalence of neutralizing antibodies depends on the virus serotype and the geographic location. Around 40% of human adults in the United States or Europe are seropositive for HAdV5, the best-studied serotype, while rates exceed 80% in some African counties [44]. Antibodies to serotypes such as HAdV26 are rare in the United States or Europe but again common in Africa [45]. Human serotypes of recombinant adenoviruses expressing the rabies virus glycoprotein, although they have yielded promising results in animal studies [49], are thus not suited for immunization of humans. Vectors based on viruses isolated from nonhuman primates, such as chimpanzees, have been generated. These viruses, which are phylogenetically closely related to human serotypes, do not circulate in the human population [55]. Most human adults thus lack neutralizing antibodies to simian adenoviruses and those that have antibodies tend to have very low titers. An E1-deleted chimpanzee adenovirus SAdV-25 (also called AdC68) expressing the rabies virus glycoprotein has been tested extensively in mice and nonhuman primates [55, 69]. The virus induces, after a single intramuscular dose, potent and sustained virus-neutralizing antibody responses, which can readily be boosted by rabies virus. Animals, including nonhuman primates, were shown to be

completely protected against challenge given more than a year after vaccination. This vaccine, which is scheduled for clinical testing, is thus highly suited for PrEP. It would provide a cost-effective alternative to the current rabies vaccine and would thus allow for more wide-spread incorporation of rabies vaccination into childhood immunization programs. As remains to be tested antibody responses could be increased by a prime-boost regimen with two heterologous chimpanzee adenovirus vectors [93], which may broaden the efficacy of the vaccine to lyssaviruses that do not belong to phylogroup 1.

Summary

The number of rabies vaccines that have undergone pre-clinical testing is impressive but only a few of those have undergone clinical testing where most were shown to be relatively ineffective. The most promising approaches right now are the adjuvanted PIKA rabies vaccine that through the addition of a TLR-3 adjuvant increases the immunogenicity of a licensed vaccine and may thereby allow for dose sparing [36]. One protein vaccine based on glycoprotein VLPs produced in baculovirus is scheduled for phase III clinical trials and although results from the earlier clinical trials have not been published thus far, one would assume that the vaccine has shown safety and immunogenicity and overall non-inferiority to current vaccines in phase I/II trials. Attenuated rabies virus is unlikely to replace current vaccines but genetically modified inactivated rabies vaccines that express two copies of the rabies virus glycoprotein may be useful for PrEP and PEP [68]. Pseudotyped viruses have undergone limited testing, where they gave promising results but concerns about toxicity in humans may hinder their transition toward clinical trials [70–72]. The above-described four types of vaccines could be used in PEP and PrEP unlike genetic vaccines that due to a delayed onset of expression of the immunogen should only be considered for PrEP. The most promising genetic vaccine is the E1-deleted SAdV-25 vector that may be sufficiently cost effective for inclusion into childhood immunization programs in highly rabies endemic areas [69].

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Assessing the Potency of Inactivated Veterinary Vaccines and Oral Live Vaccines Against Rabies

Alexandre Servat, Florence Cliquet, and Marine Wasniewski

Abstract

Vaccination of wildlife and dogs against rabies is the only efficient way to control and prevent the disease in both animals and humans. Vaccines currently in use are either inactivated (for domestic animals by injectable route) or live-attenuated or biotechnology derived (for wildlife by oral route). The objective is to vaccinate large parts of the target populations to reach a herd immunity to break the chain of infection. Quality controls on the final products are required to guarantee that these medicines are safe, stable, sterile and efficient in the target species. This chapter reviews the tests required by state regulatory authorities to assess the quality of parenteral and oral vaccines prior to their market release. The main controls focus on vaccine activity, consisting in the potency test for inactivated vaccines (NIH test) and in a virus titration for oral vaccines. A focus is also done on alternative methods to apply the 3Rs (refinement, reduction and replacement of animal use) approach in animal testing. The need for international cooperation for reaching harmonized protocols among control laboratories and for testing all produced batches of vaccines prior to their use is highlighted for ensuring consistent results.

Introduction

According to WHO, about 60,000 deaths per year are due to rabies worldwide, mostly children and almost exclusively in developing countries [1–3]. As it is a neglected disease affecting poor and vulnerable people living in isolated rural areas,

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H. C. J. Ertl (ed.), *Rabies and Rabies Vaccines*,
https://doi.org/10.1007/978-3-030-21084-7_10

the burden of the disease is underestimated. Rabies is present worldwide via two major epidemiological cycles, a canine cycle in which the dog plays the role of unique vector and sylvatic cycles where wild carnivores or bats are the vectors of the disease. Nowadays, after being eliminated in dogs, rabies persists in wildlife in developed countries [4] and remains enzootic in the dog population in developing countries, mainly in Africa and Asia [5].

Whatever the origin of rabies (i.e., domestic or sylvatic), the control of this viral disease in animals relies on the herd immunity [6]. Indeed, the chain of infection can be broken when a large part of the population is efficiently immunized. This herd immunity provides adequate protection for animals and includes those which do not have developed immunity (due to vaccine failure or non-vaccination). Numerous countries have implemented rigorous health and medical measures, including animal vaccination programs to achieve control and eventually eliminate the disease [7, 8]. To be effective, such a system should provide reliable information to confirm that the objectives have been achieved and to maintain the status "zero detected rabies case" through an effective surveillance network [9]. Historically, the control of rabies in wildlife has evolved from a simple culling operation to a more comprehensive approach involving the use of strategies for both health and medical fields [10]. Indeed, the oral vaccination of wildlife was suggested for the first time in the 1970s as an approach to control rabies, due to the first genetic experiments performed under laboratory conditions allowing to produce rabies strains with an attenuated virulence [11]. Many countries in Western, Northern, and Central Europe, which have implemented such oral vaccination programs have succeeded to control and eliminate rabies in wildlife [12–14].

Active rabies immunization is the only efficient strategy to control the disease in both humans and animals. Due to the complexity of antigens contained in the vaccines and the potential inclusion of adjuvants and other antigens (for injectable vaccines) as well as the bait matrix coating the vaccine suspension (for oral vaccines), their quality may vary from batch to batch. As a result, assessment of their activity should be requested on these batches to make sure that the level of the protective immunity is compliant to what has been demonstrated in the marketing authorization dossier.

This chapter gives an update on the state of the science for the tests in use or newly developed for assessing certain quality controls undertaken on parenteral and oral vaccines prior to their market release.

Quality Criteria of Rabies Vaccines

Veterinary vaccines have been developed to break rabies virus transmission in domestic and wild nonflying mammals with the aim to reduce human rabies cases. These vaccines are either inactivated or live-attenuated, or derived from biotechnology. All parenteral vaccines are inactivated except a canary poxvirus rabies vaccine expressing the rabies glycoprotein G that can be used for immunization of cats. Oral vaccines are either live-attenuated or biotechnology-derived vaccines.

Current inactivated rabies vaccines for veterinary use are produced from the original strain used by Pasteur in 1885 (Pasteur Virus) or from derivative strains such as Pitman-Moore, Challenge Virus Standard (CVS), Flury, Street Alabama-Dufferin (SAD), etc.

Oral vaccines are currently used for vaccination of wildlife.

The current oral vaccines could be either vaccine containing a live attenuated virus or a recombinant vaccine using vaccinia virus expressing the glycoprotein (G-protein) of rabies virus [11, 12, 15, 16]. All oral attenuated virus vaccines currently used are derived from the ERA strain (Evelyn Rokitnicki Abelseth)/SAD (Street Alabama Dufferin) with various attenuation levels after passages in cell culture [17]. Then, other recombinant vaccines were developed such as one vaccine based on a human adenovirus vector used to express the G protein of rabies virus to vaccinate wildlife [18] and another one utilizing the canine adenovirus vector as live rabies virus and the glycoprotein, as protective antigen for orally vaccinating Chinese dogs [19]. Another oral vaccine currently used in Canada is a rabies glycoprotein recombinant human adenovirus type 5 oral vaccine developed to control rabies in raccoon populations in Canada. Recently, a new oral vaccine has been developed for wildlife immunization. This new construct has for parent the SADB19 oral vaccine and contains two glycoprotein genes with two modifications at position 194 and 333 of the glycoprotein. This new vaccine was recently tested for its immunogenicity and efficacy in foxes and raccoon dogs [20, 21]. The oral vaccines are incorporated into a bait casing, whose shape, texture, and odor will attract the target species and should be free of pathogens. The bait matrix should contain a marker (generally tetracycline) to further check after bait distribution that the target animal uptakes the bait. After ingestion, this antibiotic will be incorporated into the bones and teeth and can be detected in cut teeth or jaws of killed animals. The economic aspect is also important, therefore, the bait should be produced in a standardized form and if possible locally. The bait should also be resistant to the field and storage conditions that could sometimes be extreme [22]. For safety issues, a labeling system is recommended to identify the producer, the user, and the vaccine strain used [23].

Because rabies vaccines are derived from infectious biological materials, they are consequently subjected to many in-process batch controls including identification, purity, sterility (bacteria, fungi, and mycoplasma), safety, inactivation and potency (for inactivated vaccines), and virus titer, detection of extraneous agents and biomarker stability (for oral vaccines) [24]. To limit the risk of genetic modification, virus propagation should be limited to maximally five passages from the initial stock to the final vaccine [22, 24, 25].

The vaccines must be approved by a competent authority of the country where they are used and comply with national and/or international guidelines [2, 23, 24, 26]. The major recommendations for batch tests on the final product concern safety, potency, sterility, and efficacy of the vaccine suspensions.

Vaccine strains (master seed virus) should also be genetically characterized by the vaccine producer by complete genome sequencing in order to detect any potential mutations or contaminations of the initial stock of the vaccine strain virus

[24]. Specific recommendations have been established for the bait matrix of oral vaccines regarding their attractiveness and stability in the environment and also concerning the stability of the genetic biomarker.

Potency Testing of Inactivated Rabies Vaccines

Challenge Tests on Mice

The first potency test in mice for rabies inactivated vaccines was described decades ago with the Habel test [27]. From 1953, the latter was progressively replaced by the National Institutes of Health (NIH) test [28], an in vivo method consisting of a double intraperitoneal immunization of groups of mice followed 14 days later by an intracerebral challenge using a fixed strain of rabies virus (CVS). Mice are monitored during 14 days post-challenge, and the relative potency of a test vaccine is deduced by comparing its median Effective Dose (ED_{50}) with the ED_{50} of a reference vaccine. Several variant challenge methods have been proposed, as refinement, for regulatory purposes and differ mainly in the number of vaccinations, the number of mice used per vaccine dilution and the number of dilutions [29–31]. Hence, the European Pharmacopoeia monograph 0451 dedicated to inactivated rabies vaccines for veterinary use proposes a refined NIH test using a single immunization of mice and groups of ten animals leading to a first step toward the 3Rs. Significant progresses have also been made with the routine use of anesthesia prior to challenge and the adoption of humane endpoints instead of lethality. However, the NIH test and its variants present numerous disadvantages. First of all, the virus challenge carried through the intracerebral route does not really simulate the conditions of natural exposure. Similarly, the immunization by the intraperitoneal route does not reflect the classical administration routes of rabies vaccines [32]. Taken all together, these factors may lead to a bias in the estimated potency. Furthermore, the NIH test results generate highly variable results, and a difference of up to 400% in the estimated potency is considered to be acceptable as described by the World Health Organization (WHO) [28], the World Organization for Animal Health (OIE) [26] and the European Pharmacopoeia monographs [29, 30]. Besides, the challenge potency test is quite expensive and time consuming. It uses a large number of animals (at least 120 mice for one vaccine), the majority of which (generally more than 50%) are exposed to severe pain and distress associated with the development of the rabies infection. All these issues advocate for the reduction of in vivo testing and the adoption of more ethical and reliable alternative methods that do not use laboratory animals.

Serological Potency Assay

Recently, a significant step toward the adoption of an alternative method has been taken through a collaborative study [33] organized by the European Directorate for

the Quality of Medicines (EDQM). In this study, involving 13 laboratories from 10 countries (including laboratories in the EU, Canada, and the United States), the potencies of different inactivate rabies vaccines were evaluated by using a serological potency test as an alternative to the current mouse challenge test [34]. The Serological Potency Assay (SPA) implies groups of mice immunized with the pre-diluted test vaccine or the reference standard vaccine adjusted to the minimum potency of 1 IU/dose. Blood samples from all mice are taken 14 days after immunization and the amount of rabies virus-neutralizing antibodies induced after vaccination is determined using a serum neutralization test [35, 36]. The vaccine complies if the antibody titers obtained with the test vaccine is greater than or equal to the antibody titers obtained for the reference vaccine. This technique offers several advantages—it is time effective and cost effective, and shows enhanced reliability and better reproducibility when compared to the mouse challenge test, which is known to suffer from the variability [37]. It also provides a significant 3Rs improvement, by reducing the number of animals used for testing one vaccine (around 20 mice versus 120/148 mice for the mouse challenge test). It also avoids the pain and distress of both the intracranial injection and the resulting clinical symptoms in unprotected animals. This alternative method, while not entirely eliminating the use of laboratory animals and providing only "qualitative" results, is now recognized in the European Pharmacopoeia monograph dedicated to rabies inactivated vaccine for veterinary use [30]. This is the first successful concrete step toward the replacement of the mouse challenge test and its derivatives for the batch testing of rabies vaccines in Europe.

In vitro Antigen Quantification

During the past decades, many in vitro assays for the quantification of rabies virus antigens in vaccines have been developed. These techniques must be able to discriminate between the native and highly immunogenic (trimeric) form of the glycoprotein G (which plays a key role in the induction of rabies virus neutralizing antibodies) [38] and the poorly immunogenic and soluble forms of the G protein. That is mainly the reason why the attempts to correlate the quantity of antigens in the vaccines and the protective response in mice is a great challenge. Furthermore, the hurdle is much more difficult to overcome when vaccines contain adjuvant, which is usually the case for the majority of inactivated rabies vaccines for veterinary use.

The Single Radial Immunodiffusion (SRID) test, described in 1984 [39], is based on a detergent treatment of test and reference vaccines to release the glycoprotein antigen from the rabies virus particles. Serial dilutions of both vaccines are prepared and distributed into wells in an agarose gel. The free G protein diffuses radially and binds to an antibody specific for rabies glycoprotein contained in the gel. The area of the diffusion zone is proportional to the amount of glycoprotein. The measurement and the comparison of the diffusion zones between the reference preparation and the test vaccine are used to determine the antigen content of the test vaccine. The SRID

is a rapid and inexpensive method but generally fails to correlate with the mouse challenge tests and is clearly not adapted for adjuvanted vaccines. Due to its inability to provide consistent results with final vaccine batches, the SRID has never been proposed as a possible alternative method to the NIH test, but maybe useful as an in-process test.

The Antibody Binding Test (ABT) is another rapid test that was developed in the early 1970s [40]. It involves serial dilutions of reference and test vaccines with a defined concentration of neutralizing antibodies. Unabsorbed antibodies are then detected using a fluorescent focus inhibition method. Similarly to the SRID, the ABT fails to correlate properly with the mouse challenge test. Here again, the adjuvant contained in inactivated rabies veterinary vaccines interfere in the antigen/antibody binding. The modified ABT has been widely used for in-process control to determine the antigen content after purification, concentration steps leading to the final lot. Despite recent attempts to improve further the ABT, this latter is not a suitable replacement method for the antigen quantification.

The enzyme-linked immunosorbent assay (ELISA) is an in vitro alternative approach to quantify the antigen content of rabies vaccines and possibly assess their potency. Different formats of ELISAs have been described so far [41, 42]: competition and direct assays, using the same or two different monoclonal antibodies (Mabs) for plate coating and for antigen detection, or using a polyclonal antibody for plate coating and a Mab for detection. It is now widely admitted that the glycoprotein G content may be indicative of the vaccine potency provided that the detector antibody is able to recognize the properly folded trimeric form of the G protein, which is mostly involved in the induction of rabies virus neutralizing antibodies [43]. Furthermore, ELISAs should also be able to detect subpotent batches. The ELISA is currently considered as the most relevant in vitro assay by the scientific rabies community. Several international workshops on alternatives for veterinary and human rabies vaccine testing, gathering rabies experts from industries, academia, and OMCLs (Official Medicine Control Laboratories), emphasized the importance to promote the validation of ELISAs so as to replace the current mouse potency test. Particular attention should be paid to the choice of Mabs used for the plate coating and the detection of the G protein. The most significant advances have been made for non-adjuvanted rabies vaccines. Numerous G protein-based ELISAs are currently used by manufacturers for in-process control to check the consistency of the amount of antigen in human or veterinary vaccine productions. In Japan, an IC-ELISA (Immuno-capture ELISA) is also approved for batch release of veterinary rabies vaccines that do not contain adjuvant [44]. However, efforts still need to be done to facilitate the implementation of ELISAs for the quantification of glycoprotein in adjuvanted vaccines (more especially for a final lot of rabies veterinary vaccines). To that end, the development of methods aiming to remove the adjuvant, without disrupting the recognition between Mab and glycoprotein G, is a crucial step.

Testing of Oral Rabies Vaccines

Virus Titer

It must be ensured that the vaccine titer is sufficient to cause seroconversion in immunized target animals. In Europe, a system of batch release has been set up [45]. Indeed, the EDQM has adopted the Official Control Authority Batch Release of vaccines for wildlife [46]. Therefore, before being released on the market, each batch of vaccine should be tested by an Official Medicine Control Laboratory (OMCL) for appearance and virus titer and then, based on the obtained results and the dossier provided by the manufacturer, the batch should be released by the competent authority of the country. Another important point to check is the temperatures to which the vaccine baits are subjected during transport, storage, and distribution and to ensure that they do not affect vaccinal titers. This is why it appears necessary to titrate randomly a few batches at receipt and before the starting of oral vaccination campaigns. Furthermore, as the vaccine baits are delivered on the ground, they could be subjected to some local microclimatic changes that may affect both their attractiveness to the target species and also their ability to efficiently immunize the target species. To ensure the effectiveness of oral vaccination campaigns, a protocol could be set up to collect baits on the field at different times after distribution to check their vaccination titer [10].

Currently, there is no harmonized protocol for the in vitro titration of oral vaccine baits on cell cultures since the OMCLs or testing laboratories have to follow the protocol given by each manufacturer. Each procedure for titration of oral vaccines is therefore different and has its own culture medium, cell line, method for reading (e.g., "all or nothing" method or counting fluorescent foci method) as well as its own method for titer calculation (such as Neoprobit sheet, Spearman-Kärber, or Reed and Muench methods).

Discussion

Active immunization is the only efficient strategy to control rabies in both humans and animals. Rabies inactivated and live vaccines are made from infectious material, and as such, an inherent variability may be observed in the production process. Quality controls are consequently a key element to make sure that these medicines are safe, stable, and efficient in the target species.

Injectable Parenteral Rabies Vaccines

For final products that aim to be released on the market, these controls mainly focus on the potency test. Because it has been used for decades, the mouse potency test is still perceived as the gold standard method to determine the potency of inactivated rabies vaccines. Until now, validation of any new method is conducted through the

attempt to observe a direct correlation between the protection demonstrated by the NIH potency test and the results of 3Rs alternative models. However, such direct quantitative correlation may be impossible and questionable, not only because the NIH potency test is a biological assay, but also because this latter suffers from an inherent variability [37]. As a consequence, the adoption of a pass/fail correlation to the mouse challenge test through the use of potent and subpotent batches is more adapted and recommended for successful implementation and regulatory acceptance of any 3Rs alternative method. The transition from in vivo to in vitro potency assays also requires the availability of reference materials/biologicals. Both WHO and EDQM can supply rabies vaccine standards that are calibrated through the organization of collaborative studies gathering laboratories involved in rabies vaccine controls [47–49]. These standards can be used to calibrate the internal standards. The Biological Standard Programme (BSP) has been implemented by the Council of Europe to elaborate Pharmacopoeia Reference Standards such as rabies vaccines, to standardize test methods for the quality control of biologicals, and elaborate alternative methods in order to apply the 3Rs concept to use of animals in laboratory experiments. Such programs are particularly helpful and quite important to facilitate the adoption of alternative methods, and should be duplicated whenever possible. The mutual recognition of rabies vaccine potency test results is also a crucial element. Too often still, vaccines producers are requested to submit their batches for multiple potency tests before release of their products on markets located in different parts of the world. The Official Control Authority Batch Release (OCABR) [45] adopted by some European Union Member States could serve, here again, as a source of model for regulatory bodies of non-EU countries. For each controlled batch of rabies veterinary vaccines (inactivated or live), the competent authority of a Member State issues an OCABR certificate certifying that this batch is compliant with the approved specifications laid down in the relevant monographs of the Eur. Phar. and in the relevant marketing authorization. This procedure foresees that these certificates are recognized by all members of the network to avoid duplicated tests leading to a considerable decrease in animal use. The transition from the archaic NIH test to alternative methodologies also requires overcoming the conservative positions often held by regulatory authorities, control laboratories, and industries. Such a transition may require revalidation, training, quality accreditation, purchasing new equipment that equally hinders alternatives adoption. The recent adoption of the serological potency assay in Europe for batch release of rabies inactivated veterinary vaccines shows that this psychological barrier can be circumvented.

One of the most promising approaches for complete replacement of the NIH test and its variants is the antigen quantification using ELISA tests. Considerable progress have been made for product-specific ELISAs to quantify the G protein for in-process quality controls of rabies vaccines for human or veterinary use. Nevertheless, efforts are still required to obtain tools adapted for adjuvanted final products. The selection of monoclonal antibodies specific to the native trimeric form of the glycoprotein as well as the development of methodologies to remove the adjuvant without interfering with the antibody/G protein binding are key drivers to achieve this goal.

Although still insufficient, significant steps have been taken during the last decades for the benefit of the welfare of laboratory animals used for rabies vaccines potency controls: adoption of humane end-points, use of anesthetics, refinement of the NIH potency test, implementation of the mutual recognition, adoption of the serological potency assay. Unfortunately, it is regrettable to note that many of these concrete advances are not yet part of all international regulatory requirements. This reinforces the importance of international cooperation between all stakeholders and partners involved in the quality controls of vaccines. Harmonization of techniques and protocols, sharing of the last alternative method advances are key elements toward the full replacement of laboratory animals for the potency determination test of rabies vaccines.

Oral Rabies Vaccines

Thanks to the vaccination strategy in wildlife suggested by [50], based on the use of effective oral vaccines distributed during two oral vaccination campaigns per year in autumn and spring, the situation has improved dramatically in European countries of Northern, Western, and Central Europe since 1978 [7, 14].

Since 1985, dog accessibility has been reported as the main obstacle to rabies control in dogs in many parts of the world during the mass vaccination campaigns recommended by WHO. The WHO has recognized the limitations of the parenteral route for the elimination of rabies in dogs and therefore has promoted studies on oral vaccination of dogs and the development of effective vaccines and baits [23]. This oral vaccination was considered as a new approach which, either alone or in combination with parenteral vaccination, offers the possibility of a significant increase in vaccination coverage of those dogs which cannot be handled, regardless of their ownership status. The "ideal" bait for the dog population should follow the WHO requirements [23]. The OIE has showed recently a renewed interest for oral vaccination of dogs. The OIE now endorses the concept of oral vaccination of dogs, which is included in the rabies chapter from the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2018) [26].

The oral bait should be palatable and should immediately attract the target species. Its design should preferably not attract the nontarget species, including humans. Its shape should facilitate its ingestion whatever the size and age of the target species. The vaccine liquid should be in a container (sachet or blister) that does not impact its efficacy. As underlined by WHO, it is important to keep in mind that what is appropriate for one country may not necessarily be the most appropriate for another country. For the casing, for example, it must be developed and evaluated to be the most suitable and the most attractive for the target animals present in the geographical area to be vaccinated. For example, in Mexico, the bait smells like a dog biscuit, in Egypt it smells like chicken head, and it has a fish odor for dogs in the southwest of the United States [51]. Moreover, to guarantee the stability of the virus strain in the field, the integrity of bait casings should be maintained after their distribution into the environment in different climate conditions (e.g. exposure to

sunlight, rainfall, etc), so that they do not melt or be degraded until consumption [22].

The vaccine baits should be safe for target and nontarget species. For the oral vaccines, safety is more complex to check than for parenteral vaccines. As the baits are being distributed in the field, it is necessary to ensure their safety by checking that they do not induce adverse to severe effects (such as oncogenic potential of the vaccine, potential recombination with other viruses, induction of rabies in target animals, nontarget species in the vaccination area able to eat baits as well as in nonhuman primates [2, 23]. Indeed, according to the level of attenuation of the virus strain vaccine, some oral vaccines may have a residual virulence. As this virulence may cause cases of vaccine rabies [52], it is important to characterize, from a molecular point of view, all isolated rabies viruses in a vaccination area to know if a rabies case is caused by a circulating rabies virus or the rabies vaccine. For recombinant viruses expressed in the vaccinia virus vector, it is important to check that there is no potential risk to animals, humans, and to the environment [53, 54]. As for wildlife, some tests of palatability and efficacy in target populations should be performed to ensure compliance of the product for dog populations [11, 17, 51, 55–57].

Whatever the target population, wild or canine, if oral vaccines were used to vaccinate the population, a typing of the rabies strain isolated from rabies suspected vaccinated animal should be performed in case of declaration of a rabies case [2].

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WHO Perspective on Rabies

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Abstract

Reaching zero human rabies deaths by 2030, worldwide, is an ambitious but achievable goal. Great strides have been made by countries and in international coordination to realise this but more needs to be done to scale up country leadership and political will and increase investment in programmes to operationalize proven strategies and tools. Improving access to affordable, quality vaccines, for humans and animals, remains key in preventing human rabies deaths, strengthening health systems and contributing to universal health coverage.

State of the World

Rabies is a fatal but preventable zoonotic disease responsible for the deaths of more than 59,000 people each year [1]. More than 95% of human cases are caused by rabies virus transmitted by dogs through bites or deep scratches. The majority of rabies deaths occur in Africa and Asia, and over 40% in children under the age of 15 years [1, 2]. Poor and rural populations are disproportionately affected, with the greatest disease burden borne by those who can afford it least. The knowledge and tools to eliminate rabies already exist and are proven to work: (1) mass dog vaccination to stop disease transmission at its source; (2) access to prompt, appropriate pre- (PrEP) or post-exposure prophylaxis (PEP) for people; and (3) awareness of rabies disease, and the need to vaccinate dogs and seek treatment if exposed.

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H. C. J. Ertl (ed.), *Rabies and Rabies Vaccines*,
https://doi.org/10.1007/978-3-030-21084-7_11

Currently, global data reporting for rabies are infrequent and weak. Robust surveillance and data reporting is important to quantify disease burden, target and assess impact of interventions, and encourage investment in control measures. Implementing vaccination programmes in endemic areas will improve data collection and contribute to universal health coverage, i.e. equal access to affordable, quality healthcare for all. The infrastructure required for rabies is contained in the same health system that would be strengthened to cater for basic healthcare for underserved populations. With a global target of zero human rabies deaths by 2030 ("Zero by 30"), international partners WHO, FAO, OIE, and GARC are united to meet this goal through catalysing and empowering countries to end the suffering of rabies.

Global Efforts to End the Burden of Rabies

Leadership: Global Strategy to Meet Global Goals

As rabies is a zoonotic disease, its control requires close One Health collaboration between human and animal health sectors. This is embodied internationally through the Tripartite alliance between WHO, OIE, and FAO [3, 4]. In 2015, at the Global Rabies Conference in Geneva, international stakeholders set a goal of zero dog-mediated human rabies deaths by 2030, worldwide [5]. This target was selected in accordance with the United Nations Sustainable Development Goal 3 "*ensure healthy lives and promote wellbeing for all at all ages*" and specifically target 3.3, to "*end epidemics of neglected tropical diseases by 2030*" [6]. Eliminating diseases such as rabies is also in line with target 3.8, to "*achieve universal health coverage, including financial risk protection, access to quality essential healthcare services, and access to safe, effective, quality and affordable essential medicines and vaccines for all*". Strengthening health systems to achieve universal health coverage (UHC) involves the same infrastructure required to provide appropriate, affordable, and accessible treatment for persons exposed to rabies virus. In this way, building country capacity to control rabies contributes towards the infrastructure required to treat other diseases, and improve prevention and health services overall.

Engaging Partners and Stakeholders

Bringing together experts and stakeholders from countries, academia, industry, and international organisations at the Global Conference enabled the elaboration of a global strategy to reach "Zero by 30": The Global Framework for Elimination of Dog-Mediated Human Rabies. The Global Framework identifies interventions important for rabies elimination, classifying these under five pillars of rabies control (STOP-R): sociocultural, technical, organisation, political, and resources [7]. Of common importance to each of these pillars is the need to engage others, i.e. to involve communities, governments, regional and international actors, experts, and investors in a collective, collaborative action to eliminate rabies. Through supporting

initiatives such as World Rabies Day (September 28), regional networks, and public–private partnerships, WHO engages communities, supports knowledge sharing and buy-in, and advocates for elimination [8–10].

Setting Global Norms and Standards, Monitoring Health Situations, and Trends

WHO Rabies Expert Consultations provide a similarly collaborative forum to update international recommendations on the control and prevention of rabies. These aim to use current evidence, skills, and expertise to formulate practical guidelines that are grounded within the capacity of today's health systems, whilst looking to the future to strengthen these systems for a better world [11]. This process is also informed by working groups such as the Strategic Advisory Group of Experts on Immunisation (SAGE) (see section "Updating Rabies Vaccination Guidelines"). Box 1 gives a recently updated overview, key resources on rabies vaccines and immunoglobulins (summarised in section "Improving Access to Rabies Biologicals"). Collaboration with partners such as OIE allows for harmonized guidelines between human and animal health sectors, streamlining policy development, and implementation at the national and regional levels. WHO and OIE are currently developing complementary national, regional, and global reporting systems for human (via WHO) and animal (via OIE) disease surveillance systems, to enable data sharing between sectors and a more robust, comprehensive understanding of rabies burden of disease.

Box 1 Key WHO Outputs on Rabies

WHO Position Paper on Rabies Vaccines and Rabies Immunoglobulins (2018)
http://www.who.int/wer/en/ (to be published in April 2018)
Current, endorsed, WHO recommendations on rabies immunisation.

Third WHO Expert Consultation on Rabies (2018)
LINK TO COME
Overview of expert advice and recommendations on rabies prevention and control, including on rabies vaccines (Chap. 6), and prevention of rabies in humans (Chap. 7).

Global Strategic Plan to End Human Rabies Deaths by 2030 (2018)
LINK TO COME

Revision of the WHO Position on Rabies Vaccines and Rabies Immunoglobulins (2017)

(continued)

Box 1 (continued)
http://apps.who.int/iris/bitstream/10665/259533/1/WER9248.pdf?ua=1
Background and evidence review presented to the SAGE working group in October 2017.

Rationale for Investing in the Global Elimination of Dog-Mediated Human Rabies (2015)
http://apps.who.int/iris/bitstream/10665/185195/1/9789241509558_eng.pdf

Report of the Rabies Global Conference (2015)
http://apps.who.int/iris/bitstream/10665/204621/1/WHO_HTM_NTD_NZD_2016.02_eng.pdf?ua=1

Technical Support to Build Capacity and Catalyse Change

Successful, WHO-managed proof-of-concept programmes in Asia and Africa demonstrate the feasibility of rabies elimination in different country contexts. These projects have employed novel rabies control strategies, such as stimulus packages, vaccine banks and stockpiles, and mobile phone surveillance systems to find effective, locally adapted elimination strategies [12–14]. These and other efforts are supported by WHO Collaborating Centres, who assist countries through research, technical support and training. Capacity building through regional networks in Africa (PARACON), Asia (AREB), the Americas (REDIPRA), and the Middle East (MEEREB) facilitates knowledge sharing, data collection, and implementation of coordinated regional strategies [11]. WHO, as a member of the Tripartite and with partners such as the Global Alliance for Rabies Control (GARC), is actively engaged in developing global strategy and building the case for investment in rabies elimination. Initiatives such as the Global Strategic Plan to End Human Deaths from Dog-Mediated Rabies by 2030 aim to catalyse action through leveraging existing tools and platforms, and creating an enabling environment for countries to get the job done (see section "Global Strategic Plan to End Human Deaths from Dog-Mediated Rabies By 2030")[15].

Improving Access to Rabies Biologicals

Improving access to affordable, quality rabies vaccines and immunoglobulins is a key part of the global strategy to reach "Zero by 30", and triggers national programmes. WHO is working with partners and stakeholders to facilitate this through several parallel processes.

Updating Rabies Vaccination Guidelines

The new WHO recommendations for rabies immunisation will supersede the 2010 WHO position on pre-exposure prophylaxis (PrEP) and post-exposure prophylaxis (PEP) for rabies. These updated recommendations are based on new evidence and directed by public health needs that are cost-, dose-, and time-sparing, whilst assuring safety and clinical effectiveness. In addition, new guidance on prudent use of rabies immunoglobulin (RIG) is provided.

The following sections summarise the main points of the updated WHO position as endorsed by the Strategic Advisory Group of Experts on immunisation (SAGE) at its meeting in October 2017 (Box 1).

Post-exposure Prophylaxis (PEP)

Individuals with WHO category II or III exposures should receive PEP without delay as an emergency procedure.

The WHO rabies exposure categories are:

Category I	Touching or feeding animals, licks on intact skin
Category II	Nibbling of uncovered skin, minor scratches or abrasions without bleeding, licks on broken skin
Category III	Single or multiple transdermal bites or scratches; contamination of mucous membrane with saliva from licks; exposure to bat bites or scratches

PEP consists of the following steps:

1. All bite wounds and scratches should be attended to as soon as possible after the exposure; thorough washing and flushing of the wound for approximately 15 minutes, with soap or detergent and copious amounts of water is required. Where available, an iodine-containing, or similarly viricidal, topical preparation should be applied to the wound.
2. RIG should be administered for severe category III exposures. Wounds that require suturing should be sutured loosely and only after RIG infiltration into the wound.
3. A series of rabies vaccine injections should be administered promptly after an exposure.

PEP for Rabies-Exposed Individuals of All Ages and Who Were Not Subject to Previous PrEP or PEP

- Rabies vaccines can be administered by two different routes, intradermal (ID) or intramuscular (IM), and according to different schedules.

- For adults, the vaccine should always be administered in the deltoid area of the arm; for young children (aged <2 years), the anterolateral area of the thigh is recommended.
- One ID dose is 0.1 ml of vaccine and one IM dose is an entire vial of vaccine, irrespective of the vial size.
- ID PEP regimens have cost- and dose-sparing effects, even in clinics with low patient throughput.
- The recommended WHO option is, therefore, the cost-, dose-, and time-sparing ID PEP regimen:
  - 2-site ID vaccine administrations on days 0, 3, and 7.
- The previously WHO-recommended IM PEP regimens below are still considered valid options, but may not be as cost-, dose-, or time-sparing. The feasibility of either regimen is also dependent on the clinical setting and patient preferences.
  - 1-site IM vaccine administration on days 0, 3, 7 and the fourth dose between day 14 to 28;
  - 2-site IM vaccine administration on day 0 and 1-site IM on days 7 and 21.
- Changes in rabies vaccine products and/or the route of administration during the same PEP course are acceptable, if unavoidable, to ensure PEP course completion.
- Should a vaccine dose be delayed for any reason, the PEP regimen should be resumed (not restarted).
- Individuals with documented immunodeficiency should be evaluated on a case-by-case basis and receive a complete course of ID or IM PEP, including RIG.

For Individuals with Category III Exposures and Where RIG Is Indicated

- RIG provides passive immunisation and is administered only once, as soon as possible after the initiation of PEP and not beyond day 7 after the first dose of vaccine.
- Vaccines should never be withheld, regardless of the availability of RIG.
- Correctly administered, RIG neutralises the virus at the wound site within a few hours.
- Less costly than hRIG is eRIG, both of which have shown similar clinical outcomes in preventing rabies. As eRIG products are now highly purified, skin testing before administration is unnecessary and should be abandoned.
- To confer the maximum public health benefit, WHO recommends the following:
  - The maximum dose is 20 IU (hRIG) and 40 IU (eRIG) per kg of body weight. There is no minimum dose.
  - Infiltrate as much as possible into the wound; the remainder of the calculated dose of RIG does not need to be injected IM at a distance from the wound but can be fractionated in smaller, individual syringes to be used for other patients, aseptic retention given.

- If RIG is not available, thorough, prompt wound washing, together with immediate administration of the first vaccine dose, followed by a complete course of rabies vaccine, will save up to 99% of lives.
- If a limited amount of RIG is available, RIG allocation should be prioritised for exposed patients based on the following criteria (highest priority descending):
  - Multiple bites
  - Deep wounds
  - Bites to highly innervated parts of the body, such as head, neck, hands, and genitals
  - Patients with severe immunodeficiency
  - History of biting animal indicative of confirmed or probable rabies
  - A bite or scratch or exposure of a mucous membrane by a bat that can be ascertained

PEP for Rabies-Exposed Individuals Who Can Document Previous PrEP or PEP

- No RIG is indicated
- Accelerated PEP regimens apply:
  - 1-site ID vaccine administration on days 0 and 3
  - 4-site ID vaccine administration (equally distributed over the left and right deltoids, thigh or suprascapular areas) on day 0 only
  - 1-site IM vaccine administrations on days 0 and 3
- If repeat exposure occurs (i.e. re-exposure within 3 months of completion of PEP), no PEP is recommended

Pre-exposure Prophylaxis (PrEP)

PrEP recommendations for individuals at higher risk due to occupation or for subpopulations in remote rabies-endemic settings were updated considering: (1) timely access to rabies biologicals; (2) access to rabies serological testing; (3) requirements for booster vaccination; and (4) presence of rabies in wildlife reservoirs.

PrEP makes the administration of RIG unnecessary after a bite. Rabies vaccination likely provides lifetime protection, with vaccine booster in case of an exposure. A routine PrEP booster or serology for neutralising antibody titres would be recommended only if a continued, high risk of rabies exposure remains.

- Rabies vaccines can be administered by two different routes, intradermal (ID) or intramuscular (IM), and according to different schedules.
- For adults, the vaccine should be administered in the deltoid area of the arm; for young children (aged <2 years), the anterolateral area of the thigh is recommended.
- One ID dose is 0.1 ml of vaccine and one IM dose is an entire vial of vaccine, irrespective of the vial size.

- PrEP should be considered as a large-scale intervention in remote settings that have limited access to PEP if annual dog bite incidence is >5% or vampire bat exposures prevail.
- PrEP regimens for individuals of all ages are:
  - 2-site ID vaccine administrations on days 0 and 7
  - 1-site IM vaccine administrations on days 0 and 7
- Individuals with documented immunodeficiency should be evaluated on a case-by-case basis and best receive an ID or IM PrEP regimen as above, plus a third vaccine administration between days 21 and 28. Additionally, in the event of an exposure, a complete PEP course, including RIG, is recommended.

Global Strategic Plan to End Human Deaths from Dog-Mediated Rabies By 2030

In 2015, the world called for action by setting the global goal of "Zero by 30". In response to this call, WHO, OIE, FAO, and GARC have come together as the United Against Rabies collaboration. This leverages the unique strengths and expertise of each organisation in the field of rabies control. Through developing the Global Strategic Plan, the United Against Rabies collaboration aims to provide global leadership to catalyse and empower countries to prevent human rabies deaths.

The Global Strategic Plan presents a coordinated, country-centric strategy to eliminate human rabies deaths. It integrates rabies prevention with other healthcare interventions to strengthen health systems, and engage stakeholders throughout the world in the fight to end rabies. It prioritises the societal changes needed to reach zero human rabies deaths, worldwide, into three objectives (Fig. 1).

These objectives include measures to raise awareness of rabies, conduct effective dog vaccination campaigns, and expand access to PEP and RIG through regional

Fig. 1 The three objectives of the Global Strategic Plan to End Human Deaths from Dog-Mediated Rabies by 2030 [15]

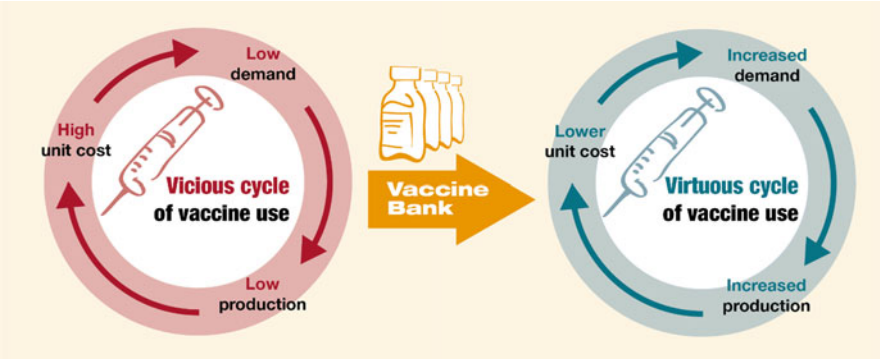

Fig. 2 Vaccine banks convert a vicious cycle of vaccine used to a virtuous one [15]

training workshops, and integrating national rabies elimination plans with health systems strengthening to contribute to universal health coverage to reach underserved populations.

Rabies Vaccine and Immunoglobulin Bank(s)

Establishing human and animal rabies biologic bank(s) is a key part of the Global Strategic Plan to catalyse increased access to rabies vaccine and immunoglobulin. This aims to ensure consistent access to quality vaccines at affordable prices, and in doing so combat shortages, allow monitoring of biological use, and improve forecasting of vaccine needs (Fig. 2). This initiative will also incentivise companies and investors to develop cost-effective vaccines and immunoglobulins, and require countries requesting biologicals to have developed strategic plans for use, forecast their own needs, and provide records of how the biologicals were used. This information will be useful to assess the impact of the bank or stockpile in improving access to rabies vaccines, and of vaccine needs in different settings. The proposal draws on mechanisms and lessons learned from previous stockpiles for human diseases such as yellow fever, meningitis and cholera, and the existing dog rabies vaccine bank of OIE [16, 17].

Future Needs

There continues to be need for vaccines that will meet programme directives, and be feasible and cost-effective to implement for community interventions. For example, easier fractionation of doses, vaccines labelled for ID use, and innovation in ID vaccine delivery technologies (e.g. microneedles) would simplify and improve uptake of cost- and dose-saving ID rabies PEP and PrEP vaccination.

Rabies vaccines that can be stored and transported outside of the conventional 2–8° C cold chain have the potential to transform vaccine delivery through increasing cost-effectiveness, efficiency, and reach of immunisation programmes. This is especially important for underserviced rural areas, where cold chains may limit access to vaccine. Progress in controlled temperature chain includes innovation in temperature monitoring of vials and standardised delivery mechanisms, however, legal liability remains a challenge.

The concept of One Health remains relevant to vaccine development, in that dog vaccine companies can learn from human vaccine companies to overcome common challenges. WHO's role is to facilitate this cross-sectoral approach, and to provide a conduit for communication between the private and public sector (i.e. manufacturers and end users).

Further recommendations on research needs for improved programmatic delivery, including on proving non-inferiority of new rabies vaccine regimens, immunisation of individuals with repeat exposures to rabies virus, efficacy and clinical outcomes of abbreviated PEP and PreP schedules, novel vaccine delivery technologies, and use of RIG are available in the updated WHO Position Paper on Rabies Vaccines and Immunoglobulins, and in Chap. 13 of the third WHO Expert Consultation on Rabies (Box 1) [11, 18].

Conclusion

Reaching zero human rabies deaths by 2030, worldwide, is an ambitious but achievable goal. Already, great strides have been made by countries and in international coordination to realise this. Improving access to affordable, quality vaccines remains key to preventing human rabies deaths, strengthening health systems and contributing to universal health coverage. As WHO Director General, Dr. Tedros Ghebreyesus, states, "Health is a human right. No one should get sick or die just because they are poor, or because they cannot access the services they need [19]". WHO is committed to empowering countries and their communities to increase access to essential health services, and end the burden of rabies.

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