Sunit K. Singh
Jens H. Kuhn *Editors*

Defense Against Biological Attacks

Volume II

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Preface 1

Biological attacks comprise a multitude of highly diverse forms of aggression using biological agents such as bacteria, toxins, and viruses. These forms range from lone-actor biocriminal activities (e.g., contamination of salad bars with bacteria, deliberate infections of others with HIV-1, murder using insulin injections) at one end of the spectrum to research and development programs that are well-funded, long-term, and clandestine nation-controlled on the other end. These latter programs may be aimed at developing deployable tactic or strategic weaponry (e.g., US, UK, Soviet biological weapons programs).

"Biodefense" has become the catch-all phrase for measures mitigating the effect of an attack with a biological agent. These measures include public and specialist education, national legislation and pathogen surveillance, international arms-control treaties and confidence-building measures, and intelligence gathering aimed at preventing the construction and deployment of biological weapons and/or their use. In addition, biodefense measures also include all responses to a biological attack, such as rapid biological agent diagnostics, emergency patient management, application of efficacious and safe medical countermeasures, and remediation of attack sites. Biodefense is therefore a highly interdisciplinary nexus for multiple subspecialties of the life sciences, humanities, and political sciences. Biodefense is a topic that has become increasingly complex; we argue that biodefense is in fact too complex for any single individual to comprehend in all of its facets. At the same time, biodefense activities and general public health measures overlap considerably. Hence, we would also argue that most public health experts can contribute significantly to biodefense and vice versa.

This two-volume book attempts to provide an overview of various priorities in biodefense in a format that is aimed to engage both laypersons and specialists. The book deliberately joins experts from various subspecialties with the hope of furthering communication between them and the readership. Volume I begins with an overview of the historical development and use of biological weapons to set the scene for past accomplishments and failures in offense research and development. Since all known nation-sponsored biological weapons programs were ultimately terminated many years ago, would such programs be organized differently and have different successes if they were undertaken today with current scientific methodologies in a different political climate? Subsequent chapters discuss whether

novel technologies, such as synthetic biology, big data analysis, and CRISPR/Cas9, could be used for nefarious purposes and whether offensive activities involving these technologies are even covered by nonproliferation treaties. Volume II provides high-level overviews of the biological agents that are most commonly associated with biodefense activities. Additional chapters present the status quo of antibacterial and antiviral therapy and diagnostic development. The book concludes with a chapter that serves as a reminder that biodefense also includes protection from attacks against nonhuman targets, a fact that is often drowned out in discussions about weapons targeting humans directly.

Of course, even a two-volume book cannot cover all aspects of biodefense, and each chapter represents only the at-times subjective assessments of individual authors rather than the consensus views of entire fields (if such views exist). However, we are proud of having recruited such a diverse set of highly renowned authors and hope that the reader shares our enthusiasm for the resulting mix of well-articulated viewpoints that demonstrate that biodefense is indeed a field of great importance.

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Preface 2

Biodefense is a nexus for multiple subspecialties of the life sciences, humanities, and political sciences. Achieving biosafe and biosecure environments for large populations requires increased interdisciplinary communication and collaboration. This book was written for policy and life science professionals, faculty, students, journalists, and laypersons to provide an overview of the multiple and often complicated facets of biodefense.

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About the Editors

Sunit K. Singh, PhD, is a Professor and Head in the Molecular Biology Unit (MBU) and the Professor Incharge of Centre for Experimental Medicine and Surgery (CEMS) at the Institute of Medical Sciences, Banaras Hindu University (BHU), Varanasi. Prof. Singh completed his PhD degree from the University of Wuerzburg, Wuerzburg, Germany, in the area of molecular infection biology and postdoctoral training from the Department of Internal Medicine, Yale University, School of Medicine, New Haven, Connecticut, USA, and the Department of Neurology, University of California Davis Medical Centre, Sacramento, California (USA). Prof. Singh also worked as a visiting faculty at the Department of Pathology, Albert Einstein College of Medicine, New York (USA); the Department of Arbovirology, Institute of Parasitology, Ceske Budejovice, Czech Republic; the Department of Pathology and Microbiology, Chonbuk National University, South Korea; and the Department of Immunology, University of Geneva, Geneva, Switzerland. Prof. Singh has been a faculty member at CSIR-Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India, and led a research group in the area of neurovirology and inflammation biology. Prof. Singh is member of various international professional organizations such as the National School of Tropical Medicine, Texas, USA, and the European Virus Bioinformatics Center, Jena, Germany. Prof. Sunit K. Singh has done seminal work in the area of neurovirology and inflammation biology. Prof. Singh's work has a very broad impact in terms of understanding the molecular mechanisms of the pathogenesis of infectious diseases in general and neurotropic viruses in particular. In addition to his original research publications in high impact factor peer-reviewed international journals, Prof. Singh has published many books in the area of infectious diseases such as

Neuroviral Infections-RNA Viruses and Retroviruses, *Neuroviral Infections-General Principles and DNA Viruses*, *Viral Hemorrhagic Fevers*, *Human Respiratory Viral Infections* from CRC Press/Taylor & Francis group, USA, Viral Infections and Global Change, *Human Emerging and Re-emerging Infectious Diseases— Vol-I and Vol-II* by Wiley Blackwell Publications, USA, and *Neglected Tropical Diseases-South Asia* by Springer, USA. Prof. Singh has been associated with many reputed peer-reviewed international journals as Associate Editor and Editorial Board Member.

Jens H. Kuhn, MD, PhD, PhD, MS, is a Research Leader at Battelle Memorial Institute, Columbus, OH, USA, tasked as the Virology Lead (Contractor) at NIH/NIAID/DCR's Biosafety Level 4 facility, the Integrated Research Facility at Fort Detrick (IRF-Frederick) in Frederick, MD, USA. Dr. Kuhn specializes in highly virulent viral human and animal pathogens. He is the author of *Filoviruses: A Compendium of 40 Years of Epidemiological, Clinical, and Laboratory Studies* (Vienna: Springer, 2008) and coauthor of *The Soviet Biological Weapons Program—A History* (Cambridge: Harvard University Press, 2012) and has studied and worked in Germany, Italy, Malta, Russia, South Africa, and South Korea. In the USA, he rotated through or worked at Harvard Medical School, Boston, MA; the Arthropod-borne Infectious Disease Laboratory (AIDL) in Fort Collins, CO; the Centers for Disease Control and Prevention (CDC) in Atlanta, GA; and the US Army Medical Research Institute of Infectious Diseases (USAMRIID) in Frederick, MD. Dr. Kuhn was the first Western scientist with permission to work in a former Soviet biological warfare facility, SRCVB "Vektor" in Siberia, Russia, within the US Department of Defense's Cooperative Threat Reduction (CTR) Program. Dr. Kuhn was a contributor to the Center for International and Security Studies at Maryland's Controlling Dangerous Pathogens Project and a member of the Center for Arms Control and Nonproliferation's CBW Scientist Working Group. He is currently a Subcommittee Chair and Member of the Executive Committee of the International Committee on Taxonomy of Viruses (ICTV), Chair of the *ICTV Bunyavirales, Filoviridae*, and *Mononegavirales* Study Groups, and a member of the ICTV *Arenaviridae*, *Bornaviridae*, *Mononegavirales*, *Nairoviridae*, and *Nyamiviridae* Study Groups. He furthermore serves as a Subject Matter Expert

for NCBI for all mononegaviruses as a member of the NCBI Genome Annotation Virus Working Group and the database RefSeq. He served or serves on the editorial boards of 10 journals and has been a peer reviewer for more than 60 journals, including *Cell, Cell Host and Microbe*, *Emerging Infectious Diseases*, *JAMA*, *The Lancet. Infectious Diseases*, *Nature*, *Nature Microbiology*, *Nature Protocols*, *PLoS Pathogens*, *Science*, and *Journal of Virology*. Dr. Kuhn was a member of the 2009–2011 US National Academy of Sciences' Committee on "Animal Models for Assessing Countermeasures to Bioterrorism Agents" and was continuously involved with AAAS's and the US State Department's bioengagement efforts in the BMENA Region, Turkey, and the NIS countries. Dr. Kuhn can be found on LinkedIn at www.linkedin.com/pub/jens-h-kuhn/1b/817/72 and on ResearchGate at https://www.researchgate.net/profile/Jens_Kuhn.

Neurotropic Viruses

1

Michael R. Holbrook

1.1 Introduction

Neurotropic viruses are those that specifically target the central nervous system (CNS). These viruses cause diseases including meningitis, encephalitis, rabies and polio-like diseases. Many of these viruses initially replicate in peripheral tissues before being introduced into the CNS. The mechanism through which some of these viruses use to penetrate the blood-brain barrier (BBB) has been established, but in many cases the specific mechanism remains unknown. The history of neurotropic viruses as potential biothreat agents is not as extensive as with many bacterial pathogens or as potentially frightening as with hemorrhagic fever-causing viruses, where disease is overt and the subject of cinematic or literary exaggeration. Neurotropic viruses cause diseases that, while not subtle in many cases, are ultimately familiar to many people. The limited "scare" factor alone decreases their value as source material for potential biothreat weapons. In their native environment, most neurotropic viruses require direct infection routes rather than contact, inhalation or ingestion, which also limits their potential for efficient infection and spread through a naïve population. Nonetheless, some neurotropic viruses have been explored for their bioweapons potential. The diseases caused by neurotropic viruses vary depending upon the specific cell type targeted by the virus. Encephalitis and meningitis are, by definition, the result of an inflammatory response that is an indirect effect of viral presence. Many of the viruses that cause encephalitis or meningitis directly infect neurons, glial cells or astrocytes to stimulate the inflammatory response. Other viruses can induce clinical encephalitis by causing pathology that allows viruses access to the brain or by stimulating an inflammatory immune response that results in clinical disease. In circumstances where clinical encephalitis

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is a secondary result of viral infection, evidence of direct viral infection of the brain may not be evident upon histological examination. For instance, rabies virus causes an overt neurological disease without causing any evident neuropathology.

Here, the principal focus is on viruses that specifically cause neurological disease through direct infection of the CNS and that have been considered or tested as potential bioweapons. A few related neurotropic viruses, such as West Nile virus, are included as examples of viruses that were introduced into naïve populations, spread and eventually became endemic. In addition to being considered source material for potential bioweapons, many of the viruses discussed here are transmitted by the bite of hematophagous arthropods (e.g., mosquitoes or ticks). With changes in the climate and the ever-increasing mobility of people and goods, the endemic range of several of these agents is increasing, and the dynamics of the natural ecological cycle is evolving, or has a significant potential to change.

1.2 Arboviruses

Arboviruses are defined as viruses that are transmitted among mammals by the bite of a hematophagous arthropod, typically a mosquito or tick, but also including sandflies and biting midges. The three principal virus taxa associated with human arboviruses are *Togaviridae* (genus *Alphavirus*), *Flaviviridae* (genus *Flavivirus*) and *Bunyavirales*. Historically the alphaviruses and flaviviruses were grouped together within the togaviruses as Group A and Group B viruses, respectively [1], but antigenic, structural and genomic differences have separated the alphaviruses and flaviviruses over time. Both the alphaviruses and flaviviruses include viruses that cause neurological disease and have been considered as source material for potential bioweapons. Bunyaviruses cause an array of diseases in humans, none of which has a specific neurological disease course.

1.2.1 Alphaviruses

Alphaviruses are small positive-sense RNA viruses that are transmitted by mosquitoes. The neurotropic alphaviruses of principal concern to human health include eastern equine encephalitis virus (EEEV), Venezuelan equine encephalitis virus (VEEV), and western equine encephalitis virus (WEEV) (Table 1.1). Other alphaviruses of significant human concern include members of the Semliki Forest complex including Semliki Forest virus, Ross River virus, chikungunya virus, and Sindbis virus. These viruses are typically associated with arthralgia rather than lethal disease but cause high morbidity and potential long-term sequelae.

1.2.1.1 Eastern Equine Encephalitis

Eastern equine encephalitis virus is found throughout the Americas and the Caribbean. The virus consist of four lineages based on geographic distribution and antigenic profile [2, 3]. Lineage I strains are found in Northern America and the

Table 1.1 Alphaviruses

Virus	Vector	Primary vertebrate hosts	Secondary vertebrate hosts	Range	Human CFR[a]
Eastern equine encephalitis virus	*Culiseta melanura* and other *Culiseta* mosquitoes	Birds	Humans, equines and other large mammals	Northern America and Caribbean (lineage I); Central and South America (lineages II–IV)	Up to 36% in infections with lineage I viruses
Western equine encephalitis virus	*Culex tarsalis*; some *Aedes* mosquitoes	Birds	Humans, equines and other large mammals	Northern, Central and South America	~4%
Venezuelan equine encephalitis virus	*Aedes taeniorhynchus*, *Aedes sollicitans* and other *Aedes* mosquitoes	Opportunistic feeder, typically mammals	NA	Northern, Central and South America	~20% in adults with neurologic disease; up to 35% in young children

[a]Case fatality rate

Caribbean while lineage II–IV strains are found in Central and South America [2]. Infection with a lineage I virus can be lethal in humans, horses, and some other domesticated animals, whereas infection with lineage II–IV viruses typically results in mild disease or subclinical infection [2, 4]. Infection of equines with lineage I EEEV is highly lethal, but these animals are also dead-end hosts and do not contribute to expansion of an outbreak, nor are they a significant risk for human infection [5]. The vectors for EEEV are primarily *Culiseta melanura* mosquitoes, but other members of the *Culiseta* genus and mosquito of other species also transmit the virus [6]. In Northern America, EEEV can cause sporadic small outbreaks that are most often first identified by cases of equine disease [5]. Human infection, while infrequent, can result in a rapidly progressing and severe neurological disease with a case fatality rate around 36% with about one third of survivors developing neurological sequelae [5]. The disease is characterized by "flu-like" symptoms and clinical signs, including fever, malaise, headache and myalgia. In some cases the infection resolves, but in others the disease progresses with signs of encephalitis such as severe headache, restlessness, drowsiness, convulsions, coma and death. Many of those who survive severe EEEV infection have long-term neurological sequelae, including severe intellectual and physical impairment that can result in death years after the acute infection has resolved [7].

In the laboratory environment, EEEV is a Risk Group 3 virus requiring BSL-3 containment, but work with this virus requires enhanced personal protective equipment (PPE) (e.g., Tyvek suit, PAPR) due to documented cases of aerosol transmission [8].

1.2.1.2 Western Equine Encephalitis Virus

Western equine encephalitis virus is found in both Northern and South America. Similar to EEEV, WEEV strains found in Northern America are epizootic and tend to cause more severe disease than their enzootic counterparts in South America [9]. After its initial isolation in California in 1930 [10], WEEV was associated with a number of large epizootics in the 1930s, 1940s, and 1950s that impacted thousands of equines and several thousand people [11]. Since that time, the number of annual cases has decreased significantly with sporadic cases of human and equine illness in western and central Northern America. There have been roughly 640 cases of human WEEV infection documented since 1964 and none since 1994 [5, 11]. Equine cases of WEEV have been reported throughout most of South America, but only a single case of WEEV infection in humans has been documented, and this case was fatal [12]. Transmission of WEEV occurs through the bite of an infected mosquito, typically of the species *Culex tarsalis*, although some *Aedes* mosquitoes have also been associated with transmission of WEEV [5, 11]. The normal enzootic cycle of WEEV is between mosquitoes and birds, including finches and house sparrows [11, 13]. Most cases of WEEV infection in humans are asymptomatic. In children, however, there is a much higher incidence of severe neurological disease with the development of encephalitis [14]. In symptomatic humans, disease is an acute onset febrile illness with common features including malaise, headache, altered mental status, and indications of meningitis [11]. Some cases develop evidence of encephalomyelitis including neck stiffness, confusion, seizures, coma and death [5, 11]. The case fatality rate for WEEV infections is around 4% [2, 5]. About one third of survivors of severe disease develop sequelae that include physical and neurocognitive disabilities. In children, the frequency of sequelae is much higher, with over 50% of children less than 10 years-old suffering long-term debilitation [11].

1.2.1.3 Venezuelan Equine Encephalitis Virus

The Venezuelan equine encephalitis antigenic complex is divided into six distinct subtypes, with VEEV encompassing subtype I [11, 15]. Subtype I VEEV is further subdivided into five serotypes (IAB, IC, ID, IE, and IF). While these subtypes were initially based on antigenicity, this division is supported by genetic differences, distribution, transmission cycle characteristics (epizootic versus enzootic), and disease phenotype. The ID and IF serotypes include only enzootic viruses that rarely cause human disease and are not known to cause a productive infection in equines [5]. The viruses in the IAB and IC subtypes are typically associated with epizootic outbreaks in both horses and humans, whereas the remaining clades are considered enzootic [5, 15]. The primary vector for the IAB and IC viruses are *Aedes taeniorhynchus* mosquitoes, but other *Aedes* mosquitoes, including those of the

species *Ae. sollicitans*, and *Psorophora* mosquitoes are also competent vectors for these viruses [2, 5, 15]. Viruses of the enzootic subtypes are typically transmitted by *Culex* mosquitoes in a cycle that includes small rodents as reservoirs or amplifying hosts [15]. In addition to humans, VEEV can infect a number of mammals and birds that can play a role as amplifying hosts during an epizootic cycle and that are critical components of the enzootic cycle [2, 15].

Infection in adult humans results in a febrile "flu-like" disease characterized by headache and myalgia. Despite the name of the virus, development of neurological disease is not common, but in those with apparent neurological disease, it can be severe with clinical signs including convulsions, disorientation, and ataxia [2, 5, 16]. The incidence of neurological complications seems to be higher in children [17]. The case fatality rate for those displaying neurological signs is around 20%, but up to 35% in children less than 5 years old [2].

1.2.1.4 Potential as Biothreat Agents

The fact that EEEV, WEEV, and VEEV are not easily disseminated in a natural environment outside of their mosquito vectors make these viruses poor options as a potential biothreat agents. However, historical references to the US and Soviet Bioweapons programs indicate that VEEV was part of both programs, [18, 19] with the Soviets also exploring the possibility of using infected mosquitoes to spread arboviruses [18]. It is likely that non-vectored transmission of these viruses, although documented in a laboratory setting through both accidental exposure and experimental aerosol transmission [20–22], would require significant effort to maintain virus viability and transmissibility in a non-controlled setting. An argument could be made for the use of these viruses as an agricultural threat, but the impact on livestock, particularly horses, would not significantly impact human health or food sources. Furthermore, as equines are dead-end hosts for the encephalitic alphaviruses, they are unlikely to contribute to the spread of disease.

1.2.1.5 Medical Countermeasures

Historically, vaccines were developed for protection against EEEV, WEEV and VEEV in the US. These vaccines were provided as IND vaccines through the Special Immunization Program (SIP) at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID). However, these vaccines are generally limited to military personnel or laboratory workers with a specific need for vaccination [23].

A number of vaccine candidates for EEEV, WEEV and VEEV have been described or are currently in development. The first effort to develop vaccines against these viruses was made in the 1940s, when researchers from the US Army developed formaldehyde-inactivated vaccines using viruses grown in mouse (EEEV and VEEV) or guinea pig (WEEV) brains [24]. Subsequent efforts used cell culture-derived virus to generate vaccines to reduce potential reactivity against brain antigens. The EEEV cell culture-based vaccine developed by USAMRIID was a formalin-inactivated vaccine [25, 26]. This vaccine seemed to be relatively well tolerated and immunogenic [25]. A similar inactivated vaccine was also developed by USAMRIID for WEEV. As with the EEEV vaccine, in a small clinical trial this

vaccine seemed to be well tolerated and immunogenic [27]. The VEEV vaccine is, perhaps, the most widely distributed of the human alphavirus vaccines. This vaccine is the live-attenuated virus TC-83, which was derived from the Trinidad donkey strain of VEEV through 83 passages in guinea pig heart cells [28]. This vaccine is known to have significant side effects in humans with around 40% of vaccinated individuals developing post-vaccination adverse reactions, some of which are severe [23]. In recent years, a number of vaccine candidates have been developed using virus chimeras, subunit vaccines, and DNA technology, along with other approaches. Some of these candidates induced a level of protective immunity, whereas others did not. The state of vaccines for EEEV, WEEV and VEEV was reviewed by Carossino et al. in 2014 and provides a reasonable description of vaccine status at that time [23]. Although some of these potential vaccines appear promising, the limited risk of infection by neurotropic alphaviruses limits the interest in developing these vaccines.

There are currently no therapeutic options available for the treatment of individuals infected by EEEV, WEEV or VEEV.

1.2.2 Flaviviruses

Two major serocomplexes of the genus *Flavivirus* consist primarily of viruses that cause neurological disease: the Japanese encephalitis virus (JEV) serocomplex and the tick-borne encephalitis virus (TBEV) serocomplex (Table 1.2). Other flaviviruses of significant concern to human health include dengue 1–4 (DENV-1–4), yellow fever (YFV), and Zika (ZIKV) viruses. Each of these viruses can, on occasion, cause neurological disease, but this is not the typical consequence of the infection. The dengue 1–4, yellow fever, and Zika viruses also do not specifically target neural tissue, whereas members of the JEV and TBEV complexes are specifically neurotropic.

1.2.2.1 Japanese Encephalitis Serocomplex

Members of the JEV serocomplex are found worldwide and are transmitted primarily by *Culex* mosquitoes. Among the viruses within the JEV serocomplex are the important human pathogens JEV, West Nile virus (WNV), St. Louis encephalitis virus (SLEV), and Murray Valley encephalitis virus (MVEV). JEV is found in Asia and island nations off the coast of Asia. In the mid-1990s, JEV was identified in an outbreak in northern Australia where the virus emerges sporadically as it appears to be transported across the Torres Strait from Indonesia [29]. WNV was originally identified in the Ugandan Protectorate in 1937 [30] and was largely restricted to Africa and Western Asia until 1999 when it was introduced into the US [31] and subsequently spread throughout the Americas. SLEV is found in the Americas and caused a number of outbreaks of encephalitis in the US between 1933 and 1990. Since the last significant outbreak in 1990, the number of identified clinical cases has been very low. MVEV is an Australian virus that was first isolated in 1951.

Table 1.2 Flaviviruses

Virus	Vector	Primary vertebrate hosts	Secondary vertebrate hosts	Range	Human CFR[a]
Japanese encephalitis virus	*Culex* spp.	Birds; domesticated and passerine	Pigs	Large parts of Asia, south into Indonesia and northern Australia	~30% of those with symptomatic disease
West Nile virus	*Culex pipiens*, *Culex quinquefasciatus* and closely related mosquitoes	Birds	Horses, humans, reptiles	Africa, Europe, Americas	Up to ~30%
Tick-borne encephalitis virus-European	*Ixodes ricinus* ticks	Small mammals/ rodents	Large mammals/ ungulates	Central and Eastern Europe	1–2%
Tick-borne encephalitis virus-Siberian	*Ixodes persulcatus* ticks	Small mammals/ rodents	Large mammals/ ungulates	Central Asia/Siberia	6–8%
Tick-borne encephalitis virus-Far Eastern	*Ixodes persulcatus* ticks	Small mammals/ rodents	Large mammals/ ungulates	Across Asia at northern latitudes	Up to ~40%

[a]Case fatality rate

Currently, the cases of human infection with MVEV are sporadic although there have been several outbreaks (most recently in 2011) [32].

Japanese Encephalitis Virus

Japanese encephalitis virus (JEV) is a mosquito-borne virus that can cause severe neurological disease in infected individuals, particularly children. Currently, there are an estimated three billion people who live in JEV endemic areas where approximately 70,000 cases occur annually with 14,000–20,500 deaths [33, 34]. The area of endemicity for JEV includes parts of 24 countries in Asia and the Asian Pacific islands [35]. JEV is subclassified into five different genotypes, with genotypes I and III being the principal genotypes circulating over the past 80 years and genotype I currently being the predominant genotype [36]. A single isolate of the fifth genotype was made in 1952 leading to speculation that this genotype was on the brink of extinction, however, recent isolates of closely related viruses suggests that the fifth JEV genotype is again circulating in Asia [37, 38]. The principal mosquito vectors of JEV include *Culex* mosquitoes that are found worldwide. The widespread distribution of potentially competent mosquitoes raises the question whether introduction of JEV into a naïve population (e.g., that of the Americas) could result in effective transmission of the virus. In Asia, the endemic cycle for JEV includes pigs and birds, including both domesticated (e.g., ducks, chickens) and wild birds. Pigs serve as an amplifying host for the virus as they can develop high titer viremias, with domesticated birds potentially playing a similar role. Passerine birds are primarily a means of virus dissemination [39, 40].

Japanese encephalitis in humans presents initially as a non-specific febrile illness that can progress to more severe disease including headache and reduced consciousness [41]. Severe neurological disease is often characterized by a dull, mask-like facies, cogwheel rigidity, and tremors, with rigidity spasms seen in patients with a poor prognosis, although a range of additional neurological signs may also be present [41]. In some instances, patients may develop acute flaccid paralysis in one or more limbs, but otherwise seem normal [42]. A percentage of these patients may develop encephalitis. Approximately one third of patients with symptomatic disease succumb to the infection. About half of the survivors of JEV infection develop long-term neurological sequelae, with many of these individuals having permanent motor neuron weakness, and some having other neurological problems including cognitive deficits [41].

West Nile Virus

West Nile virus was first isolated in 1937 in the Ugandan Protectorate [30]. In subsequent decades, this virus circulated in sylvatic cycles in Africa and parts of Western Asia, but rarely caused significant human disease. In 1999, several cases of human neurological disease in New York City were identified as being caused by St. Louis encephalitis virus, a close relative of WNV [31]. Shortly thereafter, cases of neurological disease were identified in birds at the Bronx Zoo in New York City. Virus isolates from the birds were identified by PCR as WNV and subsequent analysis of human disease samples confirmed WNV infection. Over the course of

the next decade, WNV caused thousands of cases of disease as it moved across Northern America and into Central and South America and is now considered endemic [43] with between approximately 700–5000 cases occurring annually in the US (https://www.cdc.gov/westnile/statsmaps/finalmapsdata/index.html).

West Nile virus is transmitted by the bite of an infected mosquito. The principal vectors for WNV are members of the *Cx. pipiens* complex including the *Cx. pipiens* and *Cx. quinquefasciatus* mosquitoes that are common in Asia, Africa, and the Americas [44]. The virus is maintained in its enzootic cycle between mosquitoes and susceptible bird hosts although vertical transmission in and overwintering (diapause) of mosquitoes also occurs [44]. While disease can be significant in both humans and horses, both are considered dead-end hosts and are unlikely to be significant contributors to either enzootic or epizootic cycles.

West Nile virus infection in the vast majority of humans results in a subclinical infection or a "flu-like" febrile disease (West Nile fever) that is occasionally associated with a rash [45]. West Nile fever typically resolves with little or no long-term impact. Some people, however, develop severe disease, including development of West Nile meningitis, West Nile encephalitis or a acute flaccid paralysis/polio-like illness called West Nile "poliomyelitis" [45]. West Nile meningitis typically resolves, although some people may have long-term deficits including myalgia and fatigue [46, 47]. The development of West Nile encephalitis can result in very severe disease including tremors, changes in mental status, and ataxia [48]. The case fatality rate for West Nile encephalitis has been reported up to 30% and appears to be more severe in the elderly [45, 48, 49]. Those that survive West Nile encephalitis do not typically develop loss of motor function, but may have loss of cognitive function, or may have depression or anxiety [50]. West Nile "poliomyelitis" is described as viral infection of the lower motor neurons that leads to paresis or paralysis in one or more limbs [45]. West Nile "poliomyelitis" is particularly dangerous when it affects innervation of respiratory muscles, potentially leading to respiratory failure. Indeed, more than half of the deaths associated with West Nile "poliomyelitis" are the result of respiratory failure [45]. For a more complete description of West Nile virus disease please see the review by Sevjar [45].

1.2.2.2 Tick-Borne Encephalitis Virus Serocomplex

Members of the TBEV serocomplex are transmitted by ticks and include the human pathogens TBEV, Powassan virus (POWV), and the closely related deer-tick virus (DTV), all of which commonly cause development of encephalitis or meningoencephalitis. TBEV is divided into three genotypes: Far-eastern subtype (TBEV-FE), Siberian subtype (TBEV-Sib) and the European-subtype (TBEV-Eu). Viruses of three TBEV subtypes are difficult to distinguish serologically, but can be clearly defined genetically and cause diseases that can generally be distinguished clinically. A high frequency of asymptomatic infection occurs in the case of infection with each of the three TBEV serotype viruses [51–53]. In those with symptomatic TBEV-Eu infections, the disease generally follows a biphasic disease course with a sudden onset of fever and illness that can last several days before clearing [54]. In severe cases, a second phase with neurological signs may occur. These signs include fever,

headache vomiting, disturbance of consciousness, disturbance of movement, paresis and coma, depending upon the severity of the disease [55]. The frequency of severe disease following infection with TBEV-Eu appears to be associated with age as older individuals have a much higher likelihood of developing severe disease [56]. The case fatality rate for TBE cases caused by TBEV-Eu infection is approximately 1–2% [57]. Infection with TBEV-FE can cause a very severe disease that typically is not biphasic with rapid progression to severe neurological signs. The reported case fatality rate of people infected with TBEV-FE is up to 40% with many survivors developing long-term neurological sequelae [51, 57]. Infection with TBEV-Sib can result in an intermediate type of disease that is more similar to TBEV-Eu infection, but with a higher frequency of neurologic disease and a slightly higher case fatality rate (6–8%) [54]. One notable difference of TBEV-Sib infections is that this virus has been associated with latent or chronic infections both in humans and in nonhuman primates [58–60].

The vectors for the TBEV include *Ixodes ricinus* (TBEV-Eu) and *Ix. persulcatus* (hard) ticks (TBEV-Sib and TBEV-FE) with the distribution of the viruses reflecting the ecological range of their tick vectors. TBEV-Eu is distributed in Eastern Europe, Austria, Germany, Switzerland and north into Sweden and Finland [61]. The endemic range for TBEV-FE is from far eastern-Asia and parts of Japan to the Ural mountains in Russia [62]. The TBEV-Sib is generally found in Siberia, although recent isolates of this virus subtype have been made in Finland from *Ix. ricinus* ticks [61]. The dogma is that TBEV is maintained in a sylvatic cycle between ticks and small mammals [62]. However, there is evidence that TBEV is maintained through trans-ovarial and trans-stadial transmission within ticks of the individual species [63–65]. Furthermore, transmission between ticks occurs in co-feeding ticks, with ticks feeding in a cluster transmitting virus from one tick to another [65, 66]. This "non-viremic transmission" between co-feeding ticks is thought to be an important means of viral maintenance within ticks particularly during co-feeding of nymphs and larvae [65–67]. It has also been proposed that TBEV could be maintained for extended periods of time in populations of soft ticks [68]. Transmission of TBEV to humans occurs typically through the bite of an infectious tick. Ticks can transmit TBEV at any stage of their life cycle, but transmission in smaller mammals is driven primarily by larvae and nymphs, whereas adult ticks feed principally on larger animals including ruminants and humans [69]. TBEV-Eu has been documented as being transmitted through the consumption of milk (or milk products) from infected cows, goats or sheep [70–73]. Although not clearly demonstrated for other members of the TBEV serocomplex, it is possible that other tick-borne flaviviruses could be disseminated by consumption of contaminated animal products. For further information, a comprehensive review of tick-borne encephalitis by Lindquist is recommended [54].

POWV and DTV both can cause severe encephalitis in infected humans. Although first recognized in 1958 [74], the identification of POWV cases has been very low. Since the early 2000s, the apparent frequency of POWV cases has increased. Increased concern about POWV has also led to the clear determination that DTV is distinct from POWV [75]. The potential causes of the rise in identified

POWV or DTV cases may be related to an increased abundance in the *Ixodes scapularis* (DTV) and *Dermacentor andersoni* and *Ixodes* (POWV) tick vectors, and/or increased awareness and surveillance brought about by the introduction of WNV into the US. Both POWV and DTV are fairly limited in their distribution, with most cases and virus isolates occurring in the north-central and northeastern US and southeastern Canada [76], although historical identification of POWV has occurred as far west as Colorado [77, 78].

The TBEV serocomplex also includes the notable human pathogens Omsk hemorrhagic fever virus (OHFV) and Kyasanur Forest disease virus (KFDV). Infection with either KFDV or OHFV can lead to neurological disease, but it is unclear whether either of these viruses is a truly "neurotropic" virus. The frequency of neurological cases associated with OHFV infection appears to be relatively high [79], but the few number of cases limits our understating of this disease in humans. Cases of KFDV are more often described as "viral hemorrhagic fever" [80], suggesting that neurological involvement may be the consequence of hemorrhage in the brain rather than viral tropism for neural tissue.

1.2.2.3 Potential as Biothreat Agents

As in the case of alphaviruses, the use of arthropod-borne viruses as biothreat agents is unlikely to present a significant risk to human or animal populations. Although the dissemination of JEV-infected mosquitoes is a potential risk, the technical requirements to infect and release these vectors are not trivial. Unlike the introduction of WNV into the Americas in 1999, vaccines for JEV could be rapidly deployed to mitigate widespread human infections. The potential risk of TBEV as a weapon is nominally higher due to the ability of this virus to be transmitted by ingestion. Historical accounts have shown infection with TBEV-Eu through consumption of unpasteurized milk from infected sheep or goats [70–73]. However, it is not clear how much virus is required for efficient transmission through the alimentary tract. It is likely that a significant amount of virus would be required to infect large numbers of people, and the resources and associated risk required to generate this amount of virus would be prohibitive. In addition, like JEV, vaccines for TBEV are available and are very effective (see below).

1.2.2.4 Medical Countermeasures

There are effective vaccines currently available for JEV and TBEV. There are several vaccines available for the prevention of JEV infection, including inactivated and live-attenuated virus vaccines. A live-attenuated vaccine using the SA_{14}-14-2 strain of JEV is available in many parts of Asia [81]. This vaccine requires a single initial vaccination and then a boost at 2 years, and every 6–7 years following the first boost. The Imojev vaccine is a chimeric vaccine using the viral structural protein genes from the SA_{14}-14-2 virus and cloned into the yellow fever vaccine virus 17D backbone [81]. The Imojev vaccine is available in Australia and Thailand. The inactivated Ixiaro vaccine is composed of the SA_{14}-14-2 strain [82] and is a replacement for the mouse-brain derived inactivated vaccines that have been used effectively for many years. This vaccine is available in many countries outside of

Asia, including Europe, Northern America and Australia. The Ixiaro vaccine is provided using a vaccination schedule similar to the mouse-derived vaccines, i.e., an initial series of two inoculations given 4 weeks apart, with a booster dose one year later for those with a reasonable risk of infection [82]. Specifications for provision of subsequent booster doses are not stipulated by the manufacturer, WHO [83] or the US Advisory Committee for Immunization Practices (https://www.cdc.gov/vaccines/hcp/acip-recs/vacc-specific/je.html), although historically, booster doses were given with previous inactivated JEV vaccines. There are several additional inactivated virus vaccines manufactured in various countries, with the principal difference being the virus strain used for the vaccine preparation [81, 82, 84].

The vaccines manufactured for TBEV are all inactivated viral vaccines based either on TBEV-Eu or TBEV-FE strains and using an alum adjuvant [84]. Laboratory studies suggest that the vaccines developed using TBEV-Eu may be cross-protective against the related OHFV and KFDV, but that it is only partially protective against POWV (and presumably DTV) [85]. Since the development of the TBEV vaccines, effective use of the vaccines in endemic areas has significantly reduced the total number of cases [86].

There are currently no West Nile vaccines licensed for use in humans, although there are vaccines that have been approved for use in horses [87]. The WNV horse vaccine can also be purchased as a multi-valent vaccine that includes inactivated WEEV/EEEV and VEEV (https://www.zoetisus.com/products/horses/west-nile-equine-vaccine-for-horses.aspx). The approaches used for development of a WNV vaccine have included the use of inactivated viruses, viral subunits, chimeric viruses, live-attenuated viruses and DNA-based vaccines. Some of these proposed vaccines have been tested in Phase I or Phase II clinical trials, but none are currently being evaluated in clinical settings (see Clinicaltrials.gov, search term "West Nile virus vaccine"). A comprehensive description of WNV vaccines can be found in a recent review by Amanna and Slifka [88].

There are no effective medical countermeasures available for the treatment of any flavivirus infection. Care for those infected with flaviviruses is supportive.

1.2.3 Henipaviruses

Hendra virus (HeV) was first identified in 1994 during an outbreak of severe respiratory disease of unknown etiology in horses [89]. During this initial outbreak, 14 horses died and 2 horse handlers also became infected and one of them died [89]. In both horses and humans, HeV infection resulted in severe respiratory disease. However, retrospective evaluation of a patient with fatal meningoencephalitis found that this patient had been infected with HeV [90]. Following identification of HeV as a paramyxovirus, it was determined that this virus was novel and formed its own clade within the *Paramyxoviridae*, today designated as the genus *Henipavirus* [91]. In 1996, in Malaysia, an outbreak of a disease initially thought to be Japanese encephalitis resulted in over two hundred human cases of severe disease that was frequently associated with neurological complaints [92]. During the

Table 1.3 Henipaviruses

Virus	Reservoir	Range	Human CFR[a]
Hendra virus	*Pteropus* fruit bats	Eastern coast of Australia	~57% (4 of 7 total cases)
Nipah virus	*Pteropus* fruit bats	Malaysia; Bangladesh and far-eastern India; Philippines	~54%

[a]Case fatality rate

outbreak a novel paramyxovirus, Nipah virus (NiV), was isolated and determined to be closely related to HeV (Table 1.3). The Malaysian outbreak of NiV was associated with a widespread outbreak of severe respiratory disease in pigs and resulted in the culling of over 9,00,000 pigs [92, 93]. While cases of HeV infection have been isolated to the eastern Australian coast, NiV outbreaks have occurred in Bangladesh and far northeast India [94–96] with over 600 cases documented and a case fatality rate of around 50%. In 2014, NiV RNA and virus specific antibodies were isolated from patients during an outbreak of acute neurological disease of unknown etiology in the Philippines [97]. The identification of NiV in the Philippines demonstrates that NiV is either spreading or is more broadly disseminated than previously thought. This virus has the potential to cause catastrophic outbreaks if it is introduced into relatively high-density areas.

The natural reservoirs of the henipaviruses include pteropodid bats, colloquially known as "flying foxes" [98, 99]. These animals have a broad range from the Australian coast, into the Indian subcontinent and west into Africa [100]. The range of these bats raises concern regarding the potential spread of NiV and HeV and the possibility that unidentified cases may be occurring. Transmission to other animals and humans occurs through bat excreta such as urine, but possibly also saliva and feces. In Malaysia, pigs were probably infected by consuming fruit discarded by infected bats or by coming into direct contact with bat excreta. The virus then spread among pigs through contact with pig excreta [101, 102]. In Bangladesh and India, the preponderance of human cases is thought to be the result of consumption of contaminated date palm sap [103, 104]. Measures to prevent bat access to date palm sap collection pots have been undertaken in an effort to reduce the frequency of disease [105, 106].

In humans, HeV causes a severe respiratory disease similar to influenza A virus infection with fever, drowsiness, and respiratory distress [107]. HeV infection can progress to neurological disease with motor deficits and seizures, but the extremely limited number of human cases limits our understanding of the clinical picture. Infection with NiV can result in either severe respiratory or neurological disease. During the initial outbreak in Malaysia, there was a very high incidence of neurological disease [92, 108, 109], whereas in outbreaks in Bangladesh there seems to have been a higher incidence of respiratory disease with potential late-onset or recurrent neurological disease [95]. Hamsters and ferrets experimentally infected with NiV develop severe neurological disease [110–112], whereas African green monkeys (AGM) (Grivet monkeys; *Chlorocebus aethiops*) develop a severe

hemorrhagic type disease with rapid development of respiratory complications due to edema, pulmonary infiltration, and hemorrhage [113–115]. The development of neurological disease in the AGM model seems sporadic and typically does not appear to be severe.

1.2.3.1 Potential as Biothreat Agents

Although NiV and HeV may not be considered likely source material for bioweapons targeting human, their potential impact on agriculture is significant. Since HeV seems to primarily affect horses with humans only being incidental casualties, it is unlikely that this virus would be considered in bioweapon development. However, as was seen in the initial Malaysian outbreak of NiV infection, NiV is easily transmitted between pigs in a "factory farming" environment that is now common in many countries. With the frequency of movement of pigs between farms, spread of this disease could be widespread and rapid resulting in the mass culling of animals and consequently significant impact on agricultural markets. Transmission from pigs to humans also appears to be relatively efficient and the potential for a widespread outbreak in humans working in piggeries is a significant possibility.

Although human-to-human transmission of NiV has been documented, it is not a common feature of outbreaks of NiV infection. The limited transmission of either HeV or NiV between humans limits the risk for a widespread outbreak, particularly in a nosocomial setting where proper barrier nursing techniques should be effective in preventing virus spread.

1.2.3.2 Medical Countermeasures

There are currently no antivirals or vaccines available for NiV infection, but a vaccine licensed for protection against HeV infection exists for use in horses in Australia [116]. The HeV vaccine has been shown to be cross-protective against NiV infection in the AGM model [117]. A number of vaccines for NiV are currently being developed including recombinant virus, subunit and virion-like particle (VLP)-based vaccines [116, 118]. The monoclonal Ab m102 is protective in animal studies [119] and may represent a potential option in a small-scale outbreak or laboratory exposure.

1.2.4 Lyssaviruses

Several different lyssaviruses (*Rhabdoviridae*: *Lyssavirus*) are known to cause a neurological rabies-like disease. These include some bat associated lyssaviruses and Mokola virus [120]. The most well known lyssavirus is rabies virus (RABV), a virus that causes a nearly uniformly lethal neurological disease in mammals of multiple species. Rabies virus infection is thought to result in at least 55,000 deaths worldwide annually, with the majority of these cases occurring in less developed countries. RABV is subdivided into different "terrestrial" virus variants that are associated with either foxes, dogs, raccoons or skunks; there are more than 10 variants associated with bats [121]. Although each of these variants can infect

nearly any mammal, each of the variants is maintained in its specific reservoir. The majority of human infections outside the US are the result of dog bites; canine rabies virus no longer exists in the US [121].

Rabies virus infection is typically initiated by the bite of infected animal or a significant abrasion through which the virus has access to muscle tissue. The current model for RABV infection and dissemination to the CNS requires initial replication in affected muscle tissue with migration of the virus into peripheral neurons through neuromuscular junctions using the nicotinic acetylcholine receptor (nAchR), one of the receptors for RABV [120]. Additional putative receptors for RABV include neural cell adhesion molecule (NCAM) and the p75 Nerve Growth Factor (NGF) receptor (p75NTR) [122]. Following infection of a peripheral nerve, the virus then moves to the peripheral cell body through retrograde axonal transport where is spreads trans-synapically from one neuron to another [123]. Once in the CNS, RABV appears to only infect neurons and to cause little cytopathology in infected cells [124]. The mechanisms through which RABV causes lethal disease are not clearly understood.

In humans, rabies presents initially as a febrile illness with typical signs of viral infection including malaise, headache, and irritability that can last up to 10 days [125]. The disease can develop into one of two distinct presentations, encephalitic rabies or paralytic rabies [125]. In cases of encephalitic rabies, the disease progresses through various stages of neurological involvement leading to loss of consciousness, multi-organ failure and death within 2 weeks of symptom onset. An interesting characteristic of encephalitic rabies is the development of hydrophobia in the majority of patients [125]. With paralytic rabies, disease progression is typically longer than with encephalitic rabies [126] and is characterized by weakness in the limb where the animal bite occurred with progression to quadriparesis with facial weakness and death [125]. Patients with paralytic rabies do not develop hydrophobia.

Although RABV infection is considered uniformly lethal, there have been reports of individuals surviving the infection, but most having some form of long-term sequelae [127–129]. The specific circumstances surrounding the treatment of rabies survivors varies, but the fact that some people survive what has long been held as a uniformly lethal disease provides some optimism for a cure.

1.2.4.1 Potential as Biothreat Agent

There does not appear to be evidence of RABV being tested or evaluated as a potential bioweapons agent. As RABV is not transmissible by aerosol, contact or by a means other than percutaneous injury from either a contaminated needle or infectious animal, the likelihood of dissemination is very small. The very slow development of rabies following infection would also have little impact from either a military or terror perspective. If there were a known exposure, the RABV vaccine is sufficiently available in developed countries and effective post-exposure to minimize the risk of morbidity and lethality (see below).

1.2.4.2 Medical Countermeasures

A human vaccine for RABV is available worldwide although it is not typically provided unless there is a significant risk of infection, such as to veterinarians or laboratory workers. The rabies vaccine is an inactivated virus vaccine that is given in three prophylactic doses, but is also effective when given post-exposure and before the onset of clinical signs [130]. The post-exposure regimen for high-risk exposures includes four to five vaccine doses over the course of 4 weeks with potential additional treatment with RABV-specific immunoglobulin (WHO Post-exposure guidelines: http://www.who.int/rabies/human/postexp/en/). There are no established therapies shown to be routinely affective against RABV infection. The "Milwaukee Protocol" was developed in 2004 and has been used to treat several patients with symptomatic rabies [129, 131]. This protocol puts patients in a medically induced coma and provides antiviral drugs, such as ribavirin and amantadine, with the objective of protecting the brain and allowing the development of protective immunity. Although a few patients have survived apparent RABV infection after being treated with this protocol [131], the success rate is sufficiently low that questions have been raised regarding the value of this treatment approach [132].

1.3 Summary

The use of most viruses, particularly highly virulent viruses, as bioweapons is unlikely to be effective at eliciting the widespread morbidity and mortality that many lay people envision. With the primary exception of variola virus, the viruses about which people are the most concerned are enveloped RNA viruses, as are all the viruses discussed here. Many of these viruses are susceptible to environmental conditions such as solar radiation or desiccation. These viruses are also not nearly as transmissible as many people are led to believe by the media or in books or films. Some of the viruses discussed above can be transmitted in droplets or fomites, but this is not an efficient means of transmission and, although it is possible that small, localized outbreaks could occur, the likelihood of extensive dissemination is very low. In addition, very specialized equipment and technical ability is required to produce large volumes of virus and the potential risk of accidental exposure is high. These limitations make the use of viruses, and particularly neurotropic viruses, a poor choice as bioweapons agents.

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Overview of Human Viral Hemorrhagic Fevers

2

James Logue, Martin Richter, Reed F. Johnson, Jens H. Kuhn, and Wade Weaver

2.1 Introduction

The term "viral hemorrhagic fever (VHF)" refers to an eclectic group of severe syndromes that present with apparently similar disease progression and clinical signs [1–3]. The term "VHF" for this group was first introduced by Čumakov in 1950 [4]. VHFs are typically characterized by short incubation periods followed by an acute disease phase that includes capillaropathy, coagulation abnormalities, fever, hemorrhages, and varying lethality that can surpass 50% [5, 6].

VHFs are caused by a staggering variety of viruses in taxonomically diverse animals [7–10]. This chapter focuses on human VHF-causing viruses, which are classified by the International Committee on Taxonomy of Viruses (ICTV) into the six families *Arenaviridae*, *Filoviridae*, *Flaviviridae*, *Hantaviridae*, *Nairoviridae*, and *Phenuiviridae*. These viruses typically infect arthropods and/or small mammals, whereas humans are accidental hosts (Table 2.1). After transmission to humans, the viruses are generally transmitted by direct human-to-human contact, the use of

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Table 2.1 Taxonomy of human viral hemorrhagic fever-causing pathogens and their main features

Virus (Abbreviation)	Taxonomy	Type or virus	Vector(s)	Nonhuman reservoir(s)
Alkhurma hemorrhagic fever virus (AHFV)	Family *Flaviviridae* Genus *Flavivirus*	Single-stranded, positive-sense, nonsegmented enveloped RNA virus	Sand tampans (*Ornithodoros savignyi*) and *Hyalomma dromedarii* ticks	Camels and sheep
Amur/Soochong virus (ASV)	Order *Bunyavirales* Family *Hantaviridae* Genus *Orthohantavirus*	Single-stranded, negative-sense, trisegmented enveloped RNA virus	None	Korean field mice (*Apodemus peninsulae*)
Bundibugyo virus (BDBV)	Order *Mononegavirales* Family *Filoviridae* Genus *Ebolavirus*	Single-stranded, negative-sense, nonsegmented enveloped RNA virus	Unknown	Unknown
Chapare virus (CHAPV)	Family *Arenaviridae* Genus *Mammarenavirus*	Single-stranded, ambisense, bisegmented enveloped RNA virus	Unknown	Unknown
Crimean-Congo hemorrhagic fever virus (CCHFV)	Order *Bunyavirales* Family *Nairoviridae* Genus *Orthonairovirus*	Single-stranded, negative-sense, trisegmented enveloped RNA virus	Ticks (primarily of the genus *Hyalomma*)	Cattle, dogs, goats, hares, hedgehogs, mice, ostriches, sheep
dengue viruses 1–4 (DENV 1–4)[a]	Family *Flaviviridae* Genus *Flavivirus*	Single-stranded, positive-sense, nonsegmented enveloped RNA virus	*Aedes aegypti* and *Aedes albopictus* mosquitoes	Nonhuman primates

Dobrava-Belgrade virus (DOBV)	Order *Bunyavirales* Family *Hantaviridae* Genus *Orthohantavirus*	Single-stranded, negative-sense, trisegmented enveloped RNA virus	None	Caucasus field mice (*Apodemus ponticus*), striped field mice (*Apodemus agrarius*), yellow-necked field mice (*Apodemus flavicollis*)
Ebola virus (EBOV)	Order *Mononegavirales* Family *Filoviridae* Genus *Ebolavirus*	Single-stranded, negative-sense, nonsegmented enveloped RNA virus	Unknown	Unknown
gōu virus (GOUV)	Order *Bunyavirales* Family *Hantaviridae* Genus *Orthohantavirus*	Single-stranded, negative-sense, trisegmented enveloped RNA virus	None	Brown rats (*Rattus norvegicus*), Oriental house rats (*Rattus tanezumi*), roof rats (*Rattus rattus*)
Guanarito virus (GTOV)	Family *Arenaviridae* Genus *Mammarenavirus*	Single-stranded, ambisense, bisegmented enveloped RNA virus	None	Hispid cotton rats (*Sigmodon hispidus*), short-tailed zygodonts (*Zygodontomys brevicauda*)
Hantaan virus (HTNV)	Order *Bunyavirales* Family *Hantaviridae* Genus *Orthohantavirus*	Single-stranded, negative-sense, trisegmented enveloped RNA virus	None	Striped field mice (*Apodemus agrarius*)
Junín virus (JUNV)	Family *Arenaviridae* Genus *Mammarenavirus*	Single-stranded, ambisense, bisegmented enveloped RNA virus	None	Drylands lauchas (*Calomys musculinus*)

(continued)

Table 2.1 (continued)

Virus (Abbreviation)	Taxonomy	Type or virus	Vector(s)	Nonhuman reservoir(s)
Kyasanur Forest disease virus (KFDV)	Family *Flaviviridae* Genus *Flavivirus*	Single-stranded, positive-sense, nonsegmented enveloped RNA virus	*Haemaphysalis* ticks	Bonnet macaques (*Macaca radiata*), Indomalayan vandeleurias (*Vandeleuria oleracea),* Northern Plains gray langurs (*Semnopithecus entellus*), roof rats (*Rattus rattus wroughtoni*), and other small vertebrates
Lassa virus (LASV)	Family *Arenaviridae* Genus *Mammarenavirus*	Single-stranded, ambisense, bisegmented enveloped RNA virus	None	Natal mastomys (*Mastomys natalensis*)
Lujo virus (LUJV)	Family *Arenaviridae* Genus *Mammarenavirus*	Single-stranded, ambisense, bisegmented enveloped RNA virus	None	Unknown
Machupo virus (MACV)	Family *Arenaviridae* Genus *Mammarenavirus*	Single-stranded, ambisense, bisegmented enveloped RNA virus	None	Big lauchas (*Calomys callosus*)
Marburg virus (MARV)	Order *Mononegavirales* Family *Filoviridae* Genus *Marburgvirus*	Single-stranded, negative-sense, nonsegmented enveloped RNA virus	None	Egyptian rousettes (*Rousettus aegyptiacus)*

Muju virus (MUJV)	Order *Bunyavirales* Family *Hantaviridae* Genus *Orthohantavirus*	Single-stranded, negative-sense, trisegmented enveloped RNA virus	None	Korean red-backed voles (*Myodes regulus*)
Omsk hemorrhagic fever virus (OHFV)	Family *Flaviviridae* Genus *Flavivirus*	Single-stranded, positive-sense, nonsegmented enveloped RNA virus	Ornate cow ticks (*Dermacentor reticulatus*) and other *Dermacentor* ticks	Rodents such as narrow-skulled voles (*Microtus stenocranius*), waterfowl. Muskrats (*Ondatra zibethicus*) are frequently infected but develop disease.
Puumala virus (PUUV)	Order *Bunyavirales* Family *Hantaviridae* Genus *Orthohantavirus*	Single-stranded, negative-sense, trisegmented enveloped RNA virus	None	Bank voles (*Myodes glareolus*)
Ravn virus (RAVV)	Order *Mononegavirales* Family *Filoviridae* Genus *Marburgvirus*	Single-stranded, negative-sense, nonsegmented enveloped RNA virus	None	Egyptian rousettes (*Rousettus aegyptiacus)*
Rift Valley fever virus (RVFV)	Order *Bunyavirales* Family *Phenuiviridae* Genus *Phlebovirus*	Single-stranded, ambisense, trisegmented enveloped RNA virus	*Aedes*, *Anopheles*, *Coquillettidia*, *Culex*, *Eretmapodites*, *Mansonia* mosquitoes	Cattle, goats, sheep

(continued)

Table 2.1 (continued)

Virus (Abbreviation)	Taxonomy	Type or virus	Vector(s)	Nonhuman reservoir(s)
Sabiá virus (SBAV)	Family *Arenaviridae* Genus *Mammarenavirus*	Single-stranded, ambisense, bisegmented enveloped RNA virus	Unknown	Unknown
Severe fever with thrombocytopenia syndrome virus (SFTSV)	Order *Bunyavirales* Family *Phenuiviridae* Genus *Phlebovirus*	Single-stranded, ambisense, trisegmented enveloped RNA virus	Ixodid (*Amblyomma*, *Haemaphysalis*, *Ixodes*, *Rhipicephalus*) ticks	brushtail possums, cats, hedgehogs, rodents, weasels, yaks
Seoul virus (SEOV)	Order *Bunyavirales* Family *Hantaviridae* Genus *Orthohantavirus*	Single-stranded, negative-sense, trisegmented enveloped RNA virus	None	Brown rats (*Rattus norvegicus*), roof rats (*Rattus rattus*)
Sudan virus (SUDV)	Order *Mononegavirales* Family *Filoviridae* Genus *Ebolavirus*	Single-stranded, negative-sense, nonsegmented enveloped RNA virus	Unknown	Unknown
Taï Forest virus (TAFV)	Order *Mononegavirales* Family *Filoviridae* Genus *Ebolavirus*	Single-stranded, negative-sense, nonsegmented enveloped RNA virus	Unknown	Unknown

Tula virus (TULV)	Order *Bunyavirales* Family *Hantaviridae* Genus *Orthohantavirus*	Single-stranded, negative-sense, trisegmented enveloped RNA virus	None	Common voles (*Microtus arvalis*), East European voles (*Microtus levis*), field voles (*Microtus agrestis*)
Yellow fever virus (YFV)	Family *Flaviviridae* Genus *Flavivirus*	Single-stranded, positive-sense, nonsegmented enveloped RNA virus	*Aedes aegypti* and other *Aedes* mosquitoes	Primates

[a]VHF typically occurs when a person who previously had been infected with one type of dengue virus is infected with a heterotypic type

contaminated fomites, or inhalation or other direct exposure to host animal-derived tissues or aerosolized droplets/particles contaminated with or entirely comprised of host animal secreta or excreta. For this reason, underdeveloped countries, where proper disinfection regimens, patient isolation practices, and the distribution of single-use medical supplies are lacking or are underdeveloped, experience most VHF resurgences. Additionally, individuals living in underdeveloped countries typically live in open-air houses and often work outside, which may increase exposure to reservoir hosts such as rodents and viral vectors such as mosquitos or ticks [11]. Human VHFs, most of which have specific international disease names, occur in geographically confined areas defined by the distribution of arthropod vectors and/or mammalian reservoirs of the etiologic virus [11]. Licensed prophylactic measures (vaccines) and virus-specific antiviral drugs are typically not available (Table 2.2; but also see Chap. 20). Therefore, rapid diagnosis of VHFs and identification of their causative etiological agents are critical steps following a suspected outbreak for adequate outbreak intervention via quarantine measures, education of health-care providers and affected populations, and administration of supportive treatment regimens (see Chap. 20). VHF diagnostic capabilities in the field, which are largely based on the specific and sensitive detection of viral nucleic acids (RT-PCR, next-generation sequencing, *in situ* hybridization), virus antigens (ELISA, immunohistochemistry), anti-virus antibodies (ELISA, PRNT), have vastly improved in the last decade [18].

Several VHF-causing viruses are considered potential source material for the construction of biological weapons, and some of these viruses were actively researched in past biological weapons programs [19–25]. Most of VHF-causing viruses are considered high-consequence pathogens and a great risk for public health independent of whether virus introduction occurs naturally or deliberately because of a general lack of medical countermeasures (MCMs) that could curtail virus transmission. Within the biodefense frame of this book, this chapter will provide an overview of human VHF agents that are listed as priority research pathogens by the US Centers for Disease Control and Prevention (CDC) and the US National Institute of Allergy and Infectious Diseases (NIAID). The CDC Bioterrorism Agent and NIAID Priority Pathogen lists include around 30 viruses (Table 2.2; see footnotes for caveats). These lists were created based on absence of MCMs, potential for public panic, and the lack of clinical treatment experience of health officials [26]. Most of these viruses are also tightly regulated as US Select Agents and as agents for export control (Table 2.3). Therefore, these classifications are an indirect indicator of the risk each agent is considered to pose to the US (and by extrapolation to other countries) if it emerged either naturally or via an intentional (e.g., biocriminal, bioterrorist, biowarfare) attack.

2.1.1 *Arenaviridae*

The family *Arenaviridae* currently includes three genera: *Hartmanivirus*, *Mammarenavirus*, and *Reptarenavirus* [32]. Only mammarenaviruses are known to infect humans. These viruses are commonly separated into two phylogenetic

Table 2.2 Human viral hemorrhagic fever names, distribution, and medical countermeasures

Virus (abbreviation)	WHO ICD-10 code: human disease name (abbreviation) [12]	WHO ICD-11 code: human disease name (abbreviation)[a] [13]	Geographic disease distribution [11][b]	Licensed vaccine availability	Licensed antiviral drug availability
Alkhurma hemorrhagic fever virus (AHFV)	A98.8 Other specified viral hemorrhagic fevers	1D4C: Alkhurma hemorrhagic fever (AHF)	Saudi Arabia	None	None
Amur/Soochong virus (ASV)	A98.5: epidemic hemorrhagic fever/Hantaan virus disease/ Hantan [sic] virus disease with renal manifestations/ hemorrhagic fever with renal syndrome (HFRS)/ nephropathia epidemica/Korean hemorrhagic fever/Russian hemorrhagic fever	1D62.0: hemorrhagic fever with renal syndrome (HFRS)	China, Russia, South Korea	"Hantavax" (licensed in South Korea only) [14]	Ribavirin
Bundibugyo virus (BDBV)	A98.4: Ebola virus disease (EVD)	1D60.00: Bundibugyo virus disease (BVD)	Democratic Republic of the Congo, Uganda	None	None
Chapare virus (CHAPV)	A96.8 other arenaviral hemorrhagic fevers	1D61.Y: other specified arenavirus disease	Bolivia	None	Ribavirin
Crimean-Congo hemorrhagic fever virus (CCHFV)	A98.0: Crimean-Congo hemorrhagic fever (CCHF)/ Central Asian hemorrhagic fever	1D49: Crimean-Congo hemorrhagic fever (CCHF)	Africa, Asia, Europe	Inactivated CCHFV grown in mouse brain (licensed in Bulgaria only)	Ribavirin (controversial)
dengue viruses 1–4 (DENV 1–4)	A91: dengue hemorrhagic fever (DHF)	1D20: dengue without warning signs; 1D22: severe dengue	Africa, Asia, Latin/ Central America and the Caribbean, Oceania	None	None

(continued)

Table 2.2 (continued)

Virus (abbreviation)	WHO ICD-10 code: human disease name (abbreviation) [12]	WHO ICD-11 code: human disease name (abbreviation)[a] [13]	Geographic disease distribution [11][b]	Licensed vaccine availability	Licensed antiviral drug availability
Dobrava-Belgrade virus (DOBV)	A98.5: epidemic hemorrhagic fever/Hantaan virus disease/ Hantan virus disease with renal manifestations/hemorrhagic fever with renal syndrome (HFRS)/nephropathia epidemica/Korean hemorrhagic fever/Russian hemorrhagic fever	1D62.0: hemorrhagic fever with renal syndrome (HFRS)	Europe	None	Ribavirin
Ebola virus (EBOV)	A98.4: Ebola virus disease (EVD)	1D60.01: Ebola virus disease (EVD)	Democratic Republic of the Congo, Gabon, Guinea, Republic of the Congo	None	None
gōu virus (GOUV)	A98.5: epidemic hemorrhagic fever/Hantaan virus disease/ Hantan virus disease with renal manifestations/hemorrhagic fever with renal syndrome (HFRS)/nephropathia epidemica/Korean hemorrhagic fever/Russian hemorrhagic fever	1D62.0: hemorrhagic fever with renal syndrome (HFRS)	China	None	Ribavirin
Guanarito virus (GTOV)	A96.8 other arenaviral hemorrhagic fevers	1D61.3: Venezuelan hemorrhagic fever (VeHF)	Venezuela	None	None

Hantaan virus (HTNV)	A98.5: epidemic hemorrhagic fever/Hantaan virus disease/ Hantan virus disease with renal manifestations/hemorrhagic fever with renal syndrome (HFRS)/nephropathia epidemica/Korean hemorrhagic fever/Russian hemorrhagic fever	1D62.0: hemorrhagic fever with renal syndrome (HFRS)	Korean peninsula	"Hantavax" (licensed in South Korea only) [14]	Ribavirin
Junín virus (JUNV)	A96.0: Junín (Argentinian) hemorrhagic fever	1D61.0: Argentinian hemorrhagic fever (AHF)	Argentina	"Candid-1" (licensed in Argentina only) [15]	Ribavirin
Kyasanur Forest disease virus (KFDV)	A98.2: Kyasanur Forest disease (KFD)	1D4B: Kyasanur Forest disease (KFD)	India	Formalin-inactivated KFDV vaccine (licensed in India only) [16]	None
Lassa virus (LASV)	A96.2: Lassa fever (LF)	1D61.2: Lassa fever (LF)	Africa	None	Ribavirin
Lujo virus (LUJV)	A96.8 other arenaviral hemorrhagic fevers	1D61.Y: other specified arenavirus disease	Zambia	None	Ribavirin
Machupo virus (MACV)	A96.1: Machupo (Bolivian) hemorrhagic fever	1D61.0: Bolivian hemorrhagic fever (BHF)	Bolivia	None	Ribavirin
Marburg virus (MARV)	A98.3: Marburg virus disease (MVD)	1D60.10: Marburg virus disease (MVD)	Angola, Democratic Republic of the Congo, Kenya, Uganda, Zimbabwe	None	None
Muju virus (MUJV)	A98.5: epidemic hemorrhagic fever/Hantaan virus disease/ Hantan virus disease with renal manifestations/hemorrhagic	1D62.0: hemorrhagic fever with renal syndrome (HFRS)	South Korea		Ribavirin

(continued)

Table 2.2 (continued)

Virus (abbreviation)	WHO ICD-10 code: human disease name (abbreviation) [12]	WHO ICD-11 code: human disease name (abbreviation)[a] [13]	Geographic disease distribution [11][b]	Licensed vaccine availability	Licensed antiviral drug availability
	fever with renal syndrome (HFRS)/nephropathia epidemica/Korean hemorrhagic fever/Russian hemorrhagic fever				
Omsk hemorrhagic fever virus (OHFV)	A98.1: Omsk hemorrhagic fever (OHF)	1D4A: Omsk hemorrhagic fever (OHF)	Russia	None	None
Puumala virus (PUUV)	A98.5: epidemic hemorrhagic fever/Hantaan virus disease/ Hantan virus disease with renal manifestations/hemorrhagic fever with renal syndrome (HFRS)/nephropathia epidemica/Korean hemorrhagic fever/Russian hemorrhagic fever	1D62.0: hemorrhagic fever with renal syndrome (HFRS)	Europe	None	Ribavirin
Ravn virus (RAVV)	A98.3: Marburg virus disease (MVD)	1D60.10: Marburg virus disease (MVD)	Democratic Republic of the Congo, Kenya, Uganda	None	None
Rift Valley fever virus (RVFV)	A92.4: Rift Valley fever (RFV)	1D44: Rift Valley fever (RVF)	Africa	None	None
Sabiá virus (SBAV)	A96.8 other arenaviral hemorrhagic fevers	1D61.Y: other specified arenavirus disease	Brazil	None	Ribavirin

Severe fever with thrombocytopenia syndrome virus (SFTSV)	A98.8 other specified viral hemorrhagic fevers	1D4E: severe fever with thrombocytopenia syndrome (SFTS)	China, Japan, South Korea	None	None
Seoul virus (SEOV)	A98.5: epidemic hemorrhagic fever/Hantaan virus disease/ Hantan virus disease with renal manifestations/hemorrhagic fever with renal syndrome (HFRS)/nephropathia epidemica/Korean hemorrhagic fever/Russian hemorrhagic fever	1D62.0: hemorrhagic fever with renal syndrome (HFRS)	Worldwide	"Hantavax" (licensed in South Korea only) [14]	Ribavirin
Sudan virus (SUDV)	A98.4: Ebola virus disease (EVD)	1D60.02: Sudan virus disease (SVD)	Uganda, South Sudan	None	None
Taï Forest virus (TAFV)	A98.4: Ebola virus disease (EVD)	1D60.0Y: other specified Ebola disease	Côte d'Ivoire	None	None
Tula virus (TULV)	A98.5: epidemic hemorrhagic fever/Hantaan virus disease/ Hantan virus disease with renal manifestations/hemorrhagic fever with renal syndrome (HFRS)/nephropathia epidemica/Korean hemorrhagic fever/Russian hemorrhagic fever	1D62.0: hemorrhagic fever with renal syndrome (HFRS)	Europe, Northern Asia	None	Ribavirin
Yellow fever virus (YFV)	A95: yellow fever (YF)	1D47: yellow fever (YF)	Afria, Latin/Central America and the Carribean	Live attenuated YFV vaccine "17-D" [17]	None

WHO ICD, World Health Organization International Statistical Classification of Diseases and Related Health Problems

[a]Projected codes, disease names, and abbreviations based on the latest draft version

[b]Not including human case exportations

Table 2.3 Risk classifications of human viral hemorrhagic fever-causing pathogens[a]

Virus (abbreviation)	US Centers for Disease Control and Prevention (CDC) Bioterrorism Agent/ Disease [27]	US National Institute of Allergy and Infectious Disease (NIAID) Priority Pathogen [28]	US Department of Health and Human Services (DHSS) Select Agent (US Department of Health and Human Services (DHSS) et al. [29])	Australia Group Human Pathogen for Export Control [30]	US Biocontainment Requirement [31]
Alkhurma hemorrhagic fever virus (AHFV)	Yes, Category A[b,c]	Yes, Category C[c]	No[c]	No[c]	BSL-4[c]
Amur/Soochong virus (ASV)[d]	Yes, Category A/C[e]	Yes, Category C[f]	No	No	(A)BSL3/4[g]
Bundibugyo virus (BDBV)	Yes, Category A[b]	Yes, Category A[h]	Yes (Tier 1)[i]	Yes	BSL-4
Chapare virus (CHAPV)	Yes, Category A[b]	Yes, Category A	Yes	Yes	N/i[j]
Crimean-Congo hemorrhagic fever virus (CCHFV)	Yes, Category A[b]	Yes, Category A	Yes	Yes	BSL-4
dengue viruses 1–4 (DENV 1–4)	Yes, Category A[b]	Yes, Category A	No	No	BSL-2
Dobrava–Belgrade virus (DOBV)[k]	Yes, Category A/C[e]	Yes, Category C[f]	No	Yes	(A)BSL3/4[g]
Ebola virus (EBOV)	Yes, Category A[b]	Yes, Category A[h]	Yes (Tier 1)	Yes	BSL-4
gōu virus (GOUV)	Yes, Category A/C[e]	Yes, Category C[f]	No	No	(A)BSL3/4[g]
Guanarito virus (GTOV)	Yes, Category A[b]	Yes, Category A	Yes	Yes	BSL-4
Heartland virus (HRTV)	No	Yes, Category C[l]	No	No	N/i[m]
Hantaan virus (HTNV)	Yes, Category A/C[e]	Yes, Category C[f]	No	Yes	(A)BSL3/4[g]

Junín virus (JUNV)	Yes, Category A[b]	Yes, Category A	Yes	Yes	BSL-4[n]
Kyasanur Forest virus (KFDV)	Yes, Category A[b]	Yes, Category C	Yes	Yes	BSL-4
Lassa virus (LASV)	Yes, Category A[b]	Yes, Category A	Yes	Yes	BSL-4
Lujo virus (LUJV)	Yes, Category A[b]	Yes, Category A	Yes	Yes	N/i[j]
Machupo virus (MACV)	Yes, Category A[b]	Yes, Category A	Yes	Yes	BSL-4
Marburg virus (MARV)	Yes, Category A[b]	Yes, Category A[o]	Yes (Tier 1)	Yes	BSL-4
Muju virus (MUJV)	Yes, Category A/C[e]	Yes, Category C[f]	No	No	(A)BSL3/4[g]
Omsk hemorrhagic fever virus (OHFV)	Yes, Category A[b]	Yes, Category C	Yes	Yes	BSL-4
Puumala virus (PUUV)	Yes, Category A/C[e]	Yes, Category C[f]	No	No	(A)BSL3/4[g]
Ravn virus (RAVV)	Yes, Category A[b]	Yes, Category A[o]	Yes (Tier 1)[p]	Yes	BSL-4
Reston virus (RESTV)[q]	No	Yes, Category A[h]	Yes (Tier 1)[i]	Yes	BSL-4
Rift Valley fever virus (RVFV)	Yes, Category A[b]	Yes, Category A	Yes	Yes	BSL-3[n]
Sabiá virus (SBAV)	Yes, Category A[b]	No	Yes	Yes	BSL-4
Severe fever with thrombocytopenia syndrome virus (SFTSV)	Yes, Category A[b]	Yes, Category C	No	No	N/i[m]
Seoul virus (SEOV)	Yes, Category A/C[e]	Yes, Category C[f]	No	Yes	(A)BSL3/4[g]
Sudan virus (SUDV)	Yes, Category A[b]	Yes, Category A[h]	Yes (Tier 1)[i]	Yes	BSL-4
Taï Forest virus (TAFV)	Yes, Category A[b]	Yes, Category A[h]	Yes (Tier 1)[i]	Yes	BSL-4

(continued)

Table 2.3 (continued)

Virus (abbreviation)	US Centers for Disease Control and Prevention (CDC) Bioterrorism Agent/ Disease [27]	US National Institute of Allergy and Infectious Disease (NIAID) Priority Pathogen [28]	US Department of Health and Human Services (DHSS) Select Agent (US Department of Health and Human Services (DHSS) et al. [29])	Australia Group Human Pathogen for Export Control [30]	US Biocontainment Requirement [31]
Tula virus (TULV)	Yes, Category A/C[e]	Yes, Category C[f]	No	No	(A)BSL3/4[g]
yellow fever virus (YFV)	Yes, Category A[b]	Yes, Category C	No	Yes	BSL-3[n]

(A)BSL, (animal) biosafety level; N/i, not included

[a]Shown here are only those human VHF-causing viruses that are listed by the US Centers for Disease Control and Prevention or the US National Institute of Allergy and Infectious Disease or both

[b]CDC lists "Viral hemorrhagic fevers, including. . ." as Category A. Hence all human VHF-causing agents are listed here as Category A

[c]Note that AHFV could be considered a subtype of Kyasanur Forest disease virus (KFDV)

[d]Includes Amur virus (AMRV) and Soochong virus (SOOV)

[e]Covered by CDC's "Viral hemorrhagic fevers, including. . ." as Category A Bioterrorism Agents, but included also in Category C as "Emerging infectious diseases such as . . . hantavirus [sic]"

[f]Covered by the phrase "Additional hantaviruses". Since all other hantaviruses covered by NIAID cause hantavirus (cardio)pulmonary syndrome, it is assumed here that all hemorrhagic fever-causing hantaviruses are Category C Priority Pathogens

[g]Depending on the experiment performed

[h]Interpreted here to be included in the list phrase "Ebola"

[i]Interpreted to be included in the list phrase "Ebola virus"

[j]Due to the virus' close relationship to other human VHF-causing mammarenaviruses, this virus is typically handled at BSL-4

[k]Includes Dobrava virus (DOBV), Kurkino virus (KURV), Saaremaa virus (SAAV), and Sochi virus

[l]HRTV does not cause a typical VHF and hence, is not covered in this chapter. It is listed here because it is listed under "Tickborne hemorrhagic fever virus" by NIAID

[m]Currently considered to require BSL-3 biocontainment

[n]Work with virus vaccine strains may be conducted at BSL-2

[o]Interpreted to be included in the list phrase "Marburg"

[p]Interpreted to be included in the list phrase "Marburg virus"

[q]Not known to cause disease in humans and hence not covered in this chapter

lineages, Old World and New World viruses [33]. Mammavirions are enveloped, spiked, typically 50–300 nm in diameter, and spherical, oval, or pleomorphic in shape. The mammarenaviral genome consists of two RNA segments: the S segment, encoding the nucleoprotein (NP) and spike protein (GPC); and the L segment, encoding the matrix protein Z and RNA-dependent RNA polymerase L [34]. Mammarenaviral hemorrhagic fevers are caused by at least two Old World mammarenaviruses (Lassa and Lujo) and five New World mammarenaviruses (Chapare, Guanarito, Junín, Machupo, and Sabiá). Transmission to humans primarily occurs via inhalation and ingestion of or contact with particles or droplets contaminated with or entirely comprised of excreta and secreta, blood, or tissue from murid or cricetid carrier rodents. Person-to-person transmission of mammarenaviruses is rare [34–37].

2.1.1.1 Old World Mammarenaviruses

Lassa fever (LF), caused by Lassa virus (LASV), is a VHF that was initially described in Nigeria in 1969 [38]. Since that first description, Lassa fever outbreaks have been documented across Western Africa (Guinea, Liberia, Mali, Nigeria, Sierra Leone), including a recent outbreak in Nigeria with a documented case fatality rate (CFR) of 25.4% (105 deaths out of 413 laboratory confirmed cases) from January 1 to April 15 2018 [39]. Up to 100,000–300,000 people are estimated to be infected with LASV annually, with most cases resulting in a mild, febrile illness. However, 20% of confirmed cases develop acute viral hemorrhagic fever, and approximately 5000 people succumb to disease every year [40, 41]. LF outbreaks typically trace back to human contact with the principal LASV reservoir host, the Natal mastomys (*Mastomys natalensis*) [42, 43], but person-to-person transmission also occurs. Initial clinical signs and symptoms of LF typically appear 2–16 days post-exposure and are generally nonspecific, including arthralgia, fever, headaches, and myalgia. The disease progresses to include cough, chest pain (sometimes leading to acute respiratory distress syndrome), conjunctivitis, vomiting, and diarrhea. Occasionally LF signs and symptoms include hemorrhagic manifestations (petechiae, purpura, ecchymoses, epistaxis, gastrointestinal and genitourinary bleeding) and encephalopathy (tremors, convulsions, coma) in the late stages of severe cases. Death occurs after multiorgan failure. Approximately 30% of survivors of laboratory-confirmed LF suffer from unilateral or bilateral sensorineural deafness [38, 44, 45]. Ribavirin is often used off label to treat LF, although ribavirin's overall usefulness remains under debate [46].

Lujo virus infections are caused by Lujo virus (LUJV). The only documented outbreak occurred in 2008 and affected 5 patients in South Africa and Zambia, 4 of whom died. Patients presented with nonspecific clinical signs and symptoms, including myalgia, headache, vomiting, and fever, followed by diarrhea and pharyngitis and, in terminal cases, acute respiratory distress, cerebral edema, and/or shock [47]. Due to the limited case information and fairly recent discovery of LUJV infection, little else is known about disease progression. The natural virus reservoir is yet to be identified, but due to phylogenetic relationships of LUJV, the reservoir is suspected to be a rodent.

2.1.1.2 New World Mammarenaviruses

Argentinian (Junín) hemorrhagic fever (AHF), first described in 1955 [48], is caused by Junín virus (JUNV) [49, 50]. JUNV is endemic in the Pampas region of Argentina. Occurrences of Argentinian hemorrhagic fever are generally seasonal, peaking during exposure to the natural virus reservoir, the drylands laucha (*Calomys musculinus*), during corn-harvesting season [51]. Approximately 30,000 cases of Argentinian hemorrhagic fever have been recorded (CFR ~20%), but cases have dropped dramatically after the distribution of the "Candid-1" vaccine throughout Argentina [15, 52, 53].

Bolivian (Machupo) hemorrhagic fever (BHF) was first described in 1959 in Bolivia [54] and is caused by Machupo virus (MACV) [54, 55]. MACV is generally spread through contact with food and water contaminated with excreta from the big laucha (*Calomys callosus*), which harbors the virus [56]. Human-to-human transmission is atypical. Bolivian hemorrhagic fever outbreaks are relatively rare, with approximately 1200 cases including 200 fatalities reported between 1962 and 1964 and between 2007 and 2008 [57–60]. The latest, unpublished, outbreak occurred in 2013.

"Brazilian hemorrhagic fever," caused by Sabiá virus (SBAV), resulted in only two reported naturally occurring and fatal cases, which occurred in Brazil in 1994 and 1999, respectively [61, 62]. Two other, non-fatal laboratory infections also were reported, one in Brazil in 1992 and one in the United States in 1994 [63, 64]. The close phylogenetic relationship of SBAV to other New World mammarenaviruses [33] suggests that the host of the virus is a rodent.

Chapare virus (CHAPV) caused a small VHF outbreak in Bolivia in 2003–2004. Little is known about this virus, including the natural host [65].

Venezuelan hemorrhagic fever (VeHF), caused by infection with Guanarito virus (GTOV), was officially recognized in 1989 in Brazil [66, 67]. Until 2006, a total of 618 Venezuelan hemorrhagic fever cases were reported with a fatality rate of 26% [68, 69]. An additional 86 cases of Venezuelan hemorrhagic fever were reported in 2011–2012, but how many people, if any, succumbed to infection is unclear [70]. Similar to Argentinian hemorrhagic fever, this disease predominantly affects agricultural workers during crop harvest season, when agricultural workers come into increased contact with the natural hosts of GTOV, hispid cotton rats (*Sigmodon hispidus*) and short-tailed zygodonts (*Zygodontomys brevicauda*) [71].

All New World mammarenavirus infections present similarly in humans. The incubation period lasts up to 2 weeks. Reminiscent of LF, patients first suffer from influenza-like clinical signs and symptoms, followed by abdominal pain (nausea, vomiting, constipation, diarrhea), and/or neurological impairment (vertigo, photophobia, disorientation sometimes progressing to convulsions and coma). Hemorrhagic signs develop in severe cases of infections (~30%), but blood loss is minimal. Death occurs 7–12 days after onset of disease and is typically a direct consequence of organ failure and/or shock. Various sequelae in survivors have been reported but have not yet been studied systematically. Treatment is largely symptomatic, although the use of ribavirin has been recommended in some cases [48, 65, 72–74].

2.1.2 *Bunyavirales*

The order *Bunyavirales* includes nine families comprised of 386 classified and many more unclassified viruses [32, 75]. Viruses of this order causing VHFs in humans produce enveloped and mostly spherical particles (80–120 nm) that contain trisegmented single-stranded negative-sense or ambisense RNA genomes. The small (S) segment RNA encodes the nucleoprotein (NP), the medium (M) segment RNA encodes the two virion spike proteins Gn and Gc and sometimes the nonstructural protein NSm, and the large (L) segment RNA encodes the RNA-dependent polymerase L [76]. Three families in the order (*Hantaviridae*, *Nairoviridae*, and *Phenuiviridae*) contain human VHF pathogens [11]. Human hantaviruses are carried by murid and cricetid rodents. Similar to VHF-causing arenaviruses, hantaviruses that infect humans are transmitted by contact with infected rodents or their excreta, secreta, or tissues. VHF-causing nairoviruses are maintained by ixodid ticks. Humans become infected by tick bite(s) or contact with vertebrates (or their tissues) that have been infected via tick bites. Phenuiviruses that cause human VHFs are transmitted by insects or ticks [11].

2.1.2.1 Hantaviruses

Hemorrhagic fever with renal syndrome (HFRS) is caused by several hantaviruses, most notably Amur/Soochong virus, Dobrava-Belgrade virus, gōu virus, Hantaan virus, Muju virus, Puumala virus, Seoul virus, and Tula virus [11]. HFRS was probably first described during the Warring States Period in Imperial China, followed by reports from Imperial Russia in 1913. HFRS was then recognized as a unique disease in Scandinavia, Imperial Japan, and the Soviet Union between 1930 and 1945 [77–79]. HFRS affected thousands of UN troops during the Korean War in 1951 [80]. Roughly 200,000 HFRS-infected patients are hospitalized every year from over 90 countries in Asia and Europe [81]. HFRS-causing hantaviruses persistently and subclinically infect specific rodents. Hantavirus transmission to humans occurs via contact with these rodents or their excreta, secreta, or tissues. The infection is systemic. The disease course is divided into five phases. After an incubation period of 2–4 weeks, disease begins with the febrile phase (3–7 days) characterized by influenza-like clinical signs. The disease then progresses to the hypotensive stage (~2 days of hypotension, hypoxemia, tachycardia, and thrombocytopenia), the oliguric phase (~3–7 days of renal failure with proteinuria), the diuretic phase (several weeks), and finally the convalescent phase [82–85]. The severity of HFRS is highly dependent on the causative agent, with Hantaan virus infections causing the most severe disease course and Puumala virus causing the mildest disease [86]. Ribavirin is sometimes considered a treatment option [87].

2.1.2.2 Nairoviruses

Crimean-Congo hemorrhagic fever (CCHF) was first systematically described in 1945 by Soviet researchers noticing an unusual number of deaths among 200 sick harvesters in the Steppe Region of western Crimea, now modern-day Ukraine [88]. Ticks of various species, but in particular *Hyalomma marginatum*, were

identified as the reservoirs of the etiologic agent, the nairovirus Crimean-Congo hemorrhagic fever virus (CCHFV). Today the virus is known to be endemic in Asia, Eastern Europe, and Africa [43, 89, 90]. Infections occur predominantly among peasants and other workers exposed to ticks or to tick-infested animals or their tissues (including meat products) and secretions. The incubation period typically ranges from 1 to 13 days. Disease begins suddenly with influenza-like symptoms. Though most patients improve (lethality 5–50%), a subset develops hemorrhagic signs (e.g., ecchymoses, petechiae, hematomas, hematemesis, hematuria, hemoptysis), and death commonly follows 5–14 days after disease onset. CCHFV causes the most severe hemorrhages of the VHFs and is associated with pronounced disseminated intravascular coagulation and consequent shock and organ failure [91, 92]. The benefit of treatment of patients with ribavirin is under debate [93].

2.1.2.3 Phenuiviruses

Rift Valley fever (RVF) was initially reported in 1931 when numerous sheep and cattle died or aborted in the region that is now Kenya [94]. Historically, RVF only affected humans in countries of sub-Saharan Africa and Madagascar until RVF cases were recorded for the first time in Saudi Arabia and Yemen in 2000 [95–97]. More than 300,000 cases, including at least 1220 deaths, of RFV were recorded between 1997 and 2010 [98]. RVF is caused by the Rift Valley fever virus (RVFV). RVFV is transmitted among animals and from animals to humans by a bite from an infected *Aedes* mosquito or the handling of contaminated blood or meat from cattle, goats, or sheep [97]. Though RVFV frequently causes asymptomatic infections in humans, some infections (~1%) can be severe. In these cases, following an initial incubation period ranging from 2 to 6 days, disease begins abruptly with biphasic fever and rigor, headaches, myalgia, arthralgia, nausea, vomiting, and jaundice. Hemorrhages, characterized by purpura, ecchymoses, petechiae, gastrointestinal bleeding, or bleeding from venipuncture sites, and CNS involvement are the harbingers of multiorgan failure and shock [97, 99]. Convalescence usually occurs rapidly. Ocular sequelae develop in some of the 1–20% of individuals experiencing acute ocular disease [100–102].

Severe fever and thrombocytopenia syndrome (SFTS) was first observed in 2009 in central China [103]. SFTS is caused by severe fever and thrombocytopenia syndrome virus (SFTSV), a virus now endemic to wooded environments in China, Japan, and South Korea [104]. Until 2016, 7419 cases of SFTS were reported from these three countries (with the vast majority of cases and 355 deaths occurring in China) [105]. Interestingly, the lethality of SFTS in Japan (31%) and South Korea (46%) is significantly higher than in China (7–12%) [105, 106]. SFTSV is primarily spread by *Haemaphysalis longicornis* ticks, which probably circulate the virus among mammals (goats, sheep, cattle) [107]. Humans are thought to be infected primarily through tick bites or direct contact with other infected humans. The incubation period is usually 1–2 weeks. SFTS begins with high fever, anorexia, myalgia, and lymphadenopathy and progresses to hemorrhagic signs, abdominal pain, diarrhea, vomiting, and multi-organ dysfunction [108, 109].

2.1.3 *Filoviridae*

The family *Filoviridae* currently includes the three genera *Marburgvirus*, *Ebolavirus*, and *Cuevavirus* [110]. Viruses of all genera possess negative-sense, single-stranded RNA genomes that encode seven structural proteins: nucleoprotein (NP), polymerase cofactor (VP35), transcriptional activator (VP30), glycoprotein ($GP_{1,2}$), matrix protein (VP40), a ribonucleocapsid-associated protein (VP24), and the RNA-dependent RNA polymerase (L). Cuevaviruses and ebolaviruses, in contrast to marburgviruses, additionally encode several secreted glycoproteins of unknown function [111, 112]. Human VHF-causing filoviruses belong to the genus *Marburgvirus* (Marburg virus [MARV] and Ravn virus [RAVV]) and *Ebolavirus* (Bundibugyo virus [BDBV], Ebola virus [EBOV], Sudan virus [SUDV], and Taï Forest virus [TAFV]). The VHFs caused by these viruses are clinically indistinguishable [113, 114]. Since the discovery of the filoviruses in 1967 [115] until early 2018, 31,602 cases and 13,332 deaths have been recorded over roughly 50 outbreaks in equatorial Africa (lethality ~42%). Almost all of these outbreaks were due to single introductions of filoviruses into human populations followed by direct human-to-human transmission. However, the exact mode of how filoviruses are transmitted to humans remains unclear [114, 116]. Filovirus disease begins with an incubation period of ~2–21 days, followed by influenza-like clinical signs (nausea, fever, headaches, diarrhea, maculopapular rash). Hemorrhagic signs (hematemesis, hemoptysis, melena, and hematuria), hiccups, tachypnea, CNS involvement (confusion, convulsions, meningitis, tinnitus, dysesthesias), and secondary infections generally are poor prognostic signs. Death may occur after multiorgan failure 8–16 days after infection. Survivors may suffer of a plethora of still rather undefined sequelae and (rarely) may be persistently infected [117, 118].

2.1.4 *Flaviviridae*

The family *Flaviviridae* is composed of over 70 viruses with nonsegmented, single-strand, positive-sense genomes. Flavivirions are spherical enveloped particles 40–60 nm in diameter. Flaviviruses produce at least 10 mature proteins from a single polyprotein precursor approximately 3400 amino acids in length. The structural proteins are the capsid (C), precursor membrane protein (prM), and envelope (E) proteins. The nonstructural portion of the polyprotein is processed into seven proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. Human VHF-causing flaviviruses are transmitted by mosquitoes, ticks or, in some cases, by milk or food products derived from mammals previously infected via arthropod bites [119].

Alkhurma hemorrhagic fever virus (AHFV) was first isolated from six different butchers with viral hemorrhagic fever in Saudi Arabia from 1995 to 1996 [120, 121]. Alkhurma hemorrhagic fever (AHF) is a tick-borne disease that is communicable directly via *Ornithodoros savignyi* and *Hyalomma dromedarii* tick bites or through interaction with animals or raw products of animals (predominantly camels and sheep) bitten by infected ticks [122]. From 2000 until 2011, roughly

300 cases of AHF were confirmed in Saudi Arabia. Results from original case studies of AHF indicated a CFR of up to 25–30%. More recent studies found subclinical cases to be common, and the CFR of AHF dropped to less than 1% [122–124]. The clinical signs of AHFV-infected patients are reminiscent of influenza (arthralgia, fever, myalgia, malaise leading to nausea with vomiting and/or diarrhea), but the disease may progress to severe CNS and hemorrhagic manifestations [125].

Severe dengue was first characterized during the late 1950s–1960s after outbreaks occurred in the Philippines [126, 127] and other South-eastern Asian countries [126, 128]. The disease can be attributed to sequential infections with heterotypic dengue viruses via infected *Aedes aegypti* and *Aedes albopictus* mosquitoes. Approximately 390 million human dengue virus infections occur per year, of which 96 million result in clinical manifestations [129, 130]. Only a subset of these cases (~500,000/year) progress to severe dengue. The CFR is ~2.5% [129, 131]. VHF due to dengue virus infection begins with influenza-like symptoms, then quickly deteriorates to prostration, hypotension, hemorrhagic manifestations (petechia, maculopapular rash, ecchymoses, gastrointestinal bleeding), hepatomegaly and/or liver failure, pleural effusions, ascites, and/or shock [132].

Kyasanur Forest disease virus (KFD) is a tick-borne disease first observed in 1957 in Mysore (now Karnataka) State of India [133], and has since been recorded in several other Indian states [134–136]. The causative agent of KFD, Kyasanur Forest disease virus (KFDV) [137, 138], is transmitted epizootically from *Haemaphysalis* ticks to small mammals (bats, hares, rodents), northern plains gray langurs (*Semnopithecus entellus*), bonnet macaques (*Macaca radiata*), and humans [135, 137–139]. Outbreaks of KFD occur seasonally between January to June and include ~50 to several hundred human and nonhuman primate infections per year [135, 140, 141]. Clinical signs develop 3–8 days post-exposure and include sudden onset of fever, myalgia, flushed conjunctiva, and/or hemorrhage (epistaxis, hematemesis, melena). Although most patients recover at 2 weeks post-exposure, some patients experience a neurologic disease state that includes intense cephalgia, tremors, and/or mental disturbance(s). The CFR of KFD is 2–10% [135, 138, 142].

Omsk hemorrhagic fever virus (OHFV), the etiologic agent of Omsk hemorrhagic fever (OHF), is endemic to the Kurgan, Novosibirsk, Omsk, and Tyumen Oblasts of Russia [143, 144]. The disease was first described after numerous VHF cases occurred in Omsk Oblast from 1940 to 1945 [145]. OHFV was first isolated in 1947 [146]. OHF is overall a rare disease, with 1334 cases recorded between 1945 and 1958, only sporadic cases recorded since then until 1988, and 165 cases recorded from 1988 through 1997 [143, 147]. OHFV is maintained by ornate cow ticks (*Dermacentor reticulatus*) [148], which feed on small mammals (in particular rodents and waterfowl) [143, 149, 150]. Most infections can be tracked to direct contact with common muskrats (*Ondatra zibethicus*), which are accidental OHFV hosts and which develop disease [151, 152]. After an incubation period of 2–7 days, OHF begins with rapid onset of fever, headaches, myalgia, and general malaise, accompanied by facial edema, gingivitis, and conjunctivitis. Hemorrhagic signs include epistaxis, enanthema of the soft palate, hematemesis, petechia,

conjunctival injections, and uterine hemorrhage. CNS involvement is common, including muscle rigidity, hearing impairment, taste reduction, delirium, and/or memory disruptions. Illness lasts 14–28 days, and the CFR is approximately 1–2% [153].

Yellow fever virus (YFV), the causative agent of yellow fever (YF) is one of the oldest recorded VHF-inducing agents. The first recorded likely outbreak of YF occurred on the island of Hispanola (currently Haiti and Dominican Republic) in 1495 [154]. During a 1793 outbreak in the United States, YF is thought to have been responsible for the death of 10–25% of the population of Philadelphia [154]. Despite the availability of a licensed, safe, and highly efficacious live-attenuated vaccine ("17-D", reviewed in Monath [17]), YF remains a significant public health concern. For instance, WHO estimates that as many as 17,000 severe YF cases and up to 60,000 deaths occurred in 2013 in Africa. The vast majority (≈90%) of YF cases occur in Africa; the remainder in Central and South America. YFV recently emerged in Angola and Brazil [155]. The outbreak originating in Angola began in 2015 and spread to the Democratic Republic of the Congo, Kenya, and China, resulting in 962 laboratory-confirmed cases and 393 deaths [156, 157]. The Brazilian outbreak began in December 2016 and resulted in 365 deaths among 1137 laboratory-confirmed cases by February 2018 [158, 159]. YF begins after the bite of a YFV-infected female yellow fever mosquito (primarily *Aedes aegypti*) and progresses in three phases after a 3–6 day incubation period. The acute phase (~3 days) is characterized by a sudden onset of fever with concomitant chills, headaches, nausea, myalgia, and photophobia. The remission phase (~2 days) is characterized by the disappearance of clinical signs, often followed by complete recovery. Those patients that do not recover enter the toxic phase characterized by fever, jaundice, vomiting, hemorrhagic manifestations (ecchymoses, hematemesis, hematuria, melena, petechiae), delirium, convulsions, stupor, coma, and finally death due to multiorgan failure [160].

2.2 Biodefense Considerations

Human VHF-causing viruses are highly infectious and cause severe incapacitating and frequently lethal diseases for which medical countermeasures are mostly absent. Some of these viruses, in particular Ebola virus, have entered the public conscious as alleged doomsday viruses [118]. These viruses instill public fear that, albeit scientifically often unfounded, can have profound detrimental economic effects on a population affected by only a few disease cases. Hence, most VHF-causing viruses are considered "attractive" potential source materials for the construction of biological weapons.

However, one must keep in mind that the absence of medical countermeasures for most human VHFs is due to the overall limited scientific knowledge on their etiologic agents. This same lack of knowledge manifests as a huge hurdle for biological weapons development. Most human VHF-causing agents are difficult to propagate even in small quantities in cell culture, let alone in quantities needed for weaponization (e.g., Crimean-Congo hemorrhagic fever virus, hantaviruses [20, 92]). VHF-causing viruses

are typically not highly contagious (person-to-person transmission requires direct contact and person-to-person aerosol transmission is rarely, if ever, observed). This feature suggests that VHF-causing viruses may only be source materials for weapons that have an initial devastating (economic, psychological, and/or clinical) impact, but that would not result in a self-sustaining epidemic in countries with advanced public health response systems. Reverse genetics systems are not yet available for most of these viruses, which means that the genomes of these viruses cannot be easily manipulated and therefore their perceived disadvantages as biological weapons agents cannot easily be overcome. Thus, the construction of VHF-causing virus-based biological weapons by laypersons (criminals, terrorist organizations, non-state actors) is overall unlikely.

On the other hand, well-supported national biological weapons programs have indeed focused on a handful of these viruses. For instance, Imperial Japan's biological weapons development program studied "epidemic hemorrhagic fever" (hemorrhagic fever with renal syndrome) in the 1930s on Chinese and other prisoners of war in the absence of knowledge of the etiological agents causing the disease (hantaviruses) [22]. Rift Valley fever virus was studied extensively by the US offensive biological weapons program before it was terminated in 1969 [23]. The World Health Organization (WHO) affirmed that an aerosol attack with 50-kg of the virus on a town with 500,000 inhabitants could cause as many as 35,000 infections with a 0.5% case fatality rate [161]. The US program also included dengue viruses, but the bioweaponeers were unable to grow the viruses and transmit them via small-particle aerosols, thus precluding them from further consideration [23, 162]. Yellow fever virus was an integral part of historical biological weapons programs in Canada, Germany, the USA, and the Soviet Union. In addition, North Korea was suspected to perform offensive research on the virus [19, 21, 25]. However, the existence of a widely available and highly efficacious vaccine against yellow fever virus ("17-D") [17] makes this virus a rather low priority agent for weaponization purposes.

The only know modern biological weapons program that focused on human VHF-causing viruses was the Soviet program that lasted until ~1990 [24]. The main pathogens researched for biological weapons construction were Ebola virus, Machupo virus, and Marburg virus. However, despite close to 20 years of clandestine, highly funded research and development activities, results were sobering and ultimately no VHF-causing virus-based weapon was deployed by the Soviets [24, 163].

However, considerable technological progress has been achieved since the last known nation-supported biological weapons program was terminated. Obstacles encountered in the past that prevented biological weapons construction may be overcome now or in the future. As long as safe and efficacious, widely available and globally licensed medical countermeasures are not available for VHFs, biodefense and general public health prevention measures can only rely on public education, rapid disease diagnosis and agent identification, and consequent rapid quarantine of persons with a suspected infection and isolation of infected people. Great progress has been made in all these fields over the last years, and, hence, a mass-casualty attack with VHF-causing viruses in developed countries is becoming ever less likely. However, the effect of VHF outbreaks in underdeveloped countries

that do not have established or effective biodefense/public health systems and the reaction of the public and concomitant economic effects in developed countries to an otherwise small disease outbreak should not be underestimated.

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Variola Virus: Clinical, Molecular, and Bioterrorism Perspectives

3

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3.1 Introduction

Variola virus (VARV), a species of the genus *orthopoxvirus* and the etiologic agent of smallpox, is a historical cause of immense morbidity and mortality. Smallpox is an ancient disease, described in the historical record for over 2000 years and thought to emerge from an ancestral rodent-borne poxvirus greater than 10,000 years before present [1]. Smallpox disease was a feared scourge in multiple historical civilizations and was responsible for epidemics throughout human history [2].

Notably, smallpox was the first disease for which successful prophylaxis was developed. Beginning in China in seventeenth century, children were inoculated intranasally (*i.n.*) with fresh pustules or given the clothing of an infected child, which normally resulted in a mild smallpox disease course [3]. This practice of variolation spread to the West through way of Istanbul in the eighteenth century and became widely practiced [2]. Despite the protection that variolation offered from smallpox, variolation-associated mortality was reported to be 2–3%, along with the potential for seeding of outbreaks [2].

In the late eighteenth century, however, Edward Jenner addressed the mortality issue of variolation through the scientific validation of smallpox vaccination. Jenner used cowpox virus (CPXV) lesions from a dairymaid to inoculate an 8-year-old boy. The boy experienced brief constitutional symptoms, but regained health in less than 2 weeks. Remarkably, subsequent inoculation with smallpox did not result in disease

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[4]. Jenner went on to validate this finding, and despite controversy, smallpox vaccination spread throughout Europe [4, 5]. The original source virus of the vaccine was lost to history, and during the nineteenth century, vaccination with vaccinia virus (VACV) supplanted the use of CPXV [6].

Despite the development of a vaccine, more than 300 million deaths from smallpox occurred during the twentieth century in addition to innumerable cases of debilitating disease and disfiguration [6, 7]. To address this, the World Health Organization (WHO) implemented the Smallpox Eradication Program (SEP) from 1966 to 1980, which culminated in the eradication of the disease. The success of the SEP is attributed to the absence of a zoonotic reservoir for VARV, characteristic skin lesions that identified the disease, and extraordinary international collaboration that facilitated rapid and effective ring vaccination [6]. Despite this success, concerns remain over the re-emergence or bioterrorism use of VARV or a related poxvirus.

3.2 Biological Warfare and Bioterrorism Concerns

Historical precedent for the use of smallpox in war provides rationale for modern-day concern of smallpox biowarfare. Distribution of blankets with an inoculum of smallpox to Native Americans during the colonial period resulted in immense mortality [8]. There are also reports of *orthopoxvirus* bioweapon development or use during the American Civil War, World War II, and Cold War [9–11]. Although the disease is eradicated, concerns remain over the use of VARV as a bioterrorism agent. After eradication, most public vaccination programs ceased [6]. Hence, today there are many susceptible individuals and questionable protective immunity in vaccinated individuals [12]. The large susceptible population in tandem with the stability of the virus, efficient droplet transmission, and low ID_{50} of the virus contribute to the potential for a catastrophic outbreak if VARV re-emerged in human populations [13, 14]. In addition, the increase in the use of immunosuppressive medical therapies (chemotherapeutics, corticosteroids) as well as the HIV epidemic likely places many people at high risk for severe disease if exposed or severe adverse events from vaccination [15]. Furthermore, increased international travel and urbanization elevate the risk for efficient viral spread [16, 17]. These increases, in tandem with the relatively long incubation and prodromal period of VARV, pose a significant obstacle to effective ring vaccination and contact tracing [13, 18]. Taken together, these factors all contribute to the potential for a global pandemic from an accidental or nefarious introduction of VARV into human populations.

Although VARV has been eradicated, the infective virus is retained in two official repositories. At the end of the SEP, two official stocks were created: one at the US Centers for Disease Control and Prevention (CDC) and one at the State Research Center of Virology and Biotechnology in Russia [6, 19]. The rationale for keeping these official stocks has been the subject of much discussion, though to date the World Health Assembly has postponed an official decision regarding the destruction of these stocks until May 2019 [20].

Importantly, the likelihood that these two official stocks are the only VARV in existence is slim. Information from the former Soviet Union's biological warfare program, Biopreparat, revealed that large amounts of VARV were prepared and tested for aerosol dissemination [21]. Allegations that this testing resulted in an outbreak in a community nearby were made [21–23]. Though the warfare program is now officially terminated, the fate of the smallpox samples that were not contained within the official Russian repository site during the era of Biopreparat is unknown. Adversarial groups may have obtained these stocks during or after the cessation of the biological warfare program [22, 24]. In addition, the 2014 discovery of smallpox vials at the US National Institutes of Health provided further evidence that stocks of VARV may still exist throughout the world, outside of the two official repositories [25].

In addition to the threat of natural VARV located outside the official repositories, increasing molecular capabilities and availability of genomic information may permit the design and synthesis of more virulent or vaccine-resistant VARV [26]. The recent synthesis of infectious horsepox virus from commercially synthesized DNA underscores the possible re-emergence of VARV [20, 27]. Moreover, increasing information regarding VARV pathogenesis may allow for genetic modification of other orthopoxviruses that normally have low virulence in humans [28–30]. Finally, there have been speculations about possible VARV recombination experiments with other virulent bioterrorism agents, though the feasibility of these experiments has been questioned [21].

Likewise, the progenitor poxviruses that gave rise to VARV may produce novel, possibly zoonotic, human-tropic poxviruses [31]. Known zoonotic orthopoxviruses, such as monkeypox virus (MPXV), also pose a threat. MPXV can cause smallpox-like disease with a significant, albeit lesser, mortality rate [32, 33]. Importantly, MPXV can be transmitted zoonotically with limited human-to-human transmission. Indeed, outbreaks of MPXV infections have occurred in the Democratic Republic of the Congo, Sudan, and United States [32, 34, 35]. Furthermore, MPXV is stable and can be easily produced in cell culture systems [36] and may have been weaponized by the Soviet Union [21].

Taken together, the (1) historical precedent, (2) large susceptible human population, (3) high transmissibility, (4) existence of unofficial stocks of VARV, (5) increased dissemination of information and technology potentially capable of producing infectious VARV, (6) threat of other orthopoxviruses, and (7) increased prevalence of terrorism make a smallpox outbreak a present-day possibility. Several modeling efforts have demonstrated that infection would spread rapidly and may be hard to control depending on the scale of the introduction, even with ring vaccination and contact tracing efforts [18, 37–39]. Given that the use of VARV or a related agent for bioterrorism purposes is a legitimate concern, understanding smallpox disease pathogenesis and countermeasure development are key priorities. Since the eradication of smallpox, expanded molecular biology capabilities and animal models have furthered our understanding of the virus and disease pathogenesis. In this chapter, we address these advances and provide perspectives on their relevance to human disease. Moreover, we provide an overview

of smallpox virology, clinical disease, pathogenesis, diagnostics, prophylaxis, and clinical interventions.

3.3 Virology

3.3.1 Classification

Poxviruses infect both vertebrates and invertebrates and cause an array of important diseases in humans and animals [40]. Subfamilies of *Poxviridae* include *Entomopoxvirinae* and *Chordopoxvirinae*. Of particular interest is the *Chordopoxvirinae* subfamily, which encompasses ten genera. Of these, members of the *Parapoxvirus*, *Molluscipoxvirus*, *Yatapoxvirus*, and *orthopoxvirus* genera are known to give rise to active infections within the human population [40]. The *orthopoxvirus* genus includes VARV, MPXV, CPXV, and VACV. This genus has high antigenic similarity, which was important for smallpox eradication and possible cross-protection against other members of the *orthopoxvirus* genus with the smallpox vaccine [41]. Importantly, while most poxvirus species are zoonotic, the only known reservoir for VARV is humans [40].

3.3.2 Morphology

Poxviruses, the largest known animal viruses, range in size from 200 to 400 nm [40]. Notably, they can be visualized using light microscopy [42]. The viral core is biconcave and contains the DNA genome, DNA-dependent RNA polymerase, and enzymes necessary for particle uncoating [42]. A layer of thin rod-like prominences known as the palisade layer encircles the core. Two proteinaceous lateral bodies flank the concave portions of the core and associated palisade layer [43]. A membrane surrounds the core and lateral bodies, giving the viral particle its oval to brick shape. The membrane surrounding the core is a single lipid membrane [44]. The virion may or may not obtain another membrane prior to exiting the cell, which will be discussed in the life cycle section.

3.3.3 Genome

Within the biconcave core resides a linear double-stranded viral DNA genome. Approximately 130–300 kbp in length, poxvirus genomes are closed via hairpin loops of complimentary AT-rich regions [43, 45, 46]. The termini of the genomes are also flanked by inverted repeats containing functional open reading frames (ORFs) [41]. Within the genomes, highly conserved structural genes, responsible for the conserved architectural structure of poxviruses, are aggregated in the middle of the genome [43]. The genomes harbor approximately 200 genes and hundreds of functional ORFs. 90 of these genes are conserved across all chordopoxviruses and

encode structural proteins and factors required for viral reproduction [47, 48]. Importantly, these epitopes are highly conserved and may enable cross-protection using VACV-based vaccines for orthopoxviruses of multiple species.

The remaining unique ORFs found at the terminal ends of the viral genome can encode a diverse array of virulence factors and other proteins. This is thought to confer distinct tropism, immune evasion, virulence, and pathogenic properties to each poxvirus [43]. Interestingly, VARV has a limited number of genes at the terminal ends of the genome, a narrow host range, and high pathogenicity. In contrast, less pathogenic orthopoxviruses such as CPXV have more genes at the termini of the genome, a broad host range, and low pathogenicity. Thus, further exploration of the relationship between the genes at the terminal end of the genome, host range, and pathogenicity is warranted [47, 49, 50].

3.3.4 Life Cycle

Much of the knowledge about the poxvirus life cycle has been gained through the extensive study of VACV, which shares a substantial amount of genetic similarity with VARV [46, 47]. Although an in-depth and thorough exploration into the viral life cycle of VACV has been carried out, the particulars of the VARV life cycle remain relatively unknown. Thus, the life cycle described here is based primarily upon studies using VACV. As such, it is imperative to conduct VARV specific studies in order to characterize the unique replicative and life cycle features of VARV that may contribute to its unique pathogenicity (Fig. 3.1).

3.3.5 Life Cycle: Entry

Poxvirus virions can be differentiated based on the nature and number of their biological membranes, and these differences are of key importance for entry. Lipid membranes are acquired throughout various stages of the viral life cycle, generating morphologically distinct virions that have distinct surface markers. The building block of all morphologies is the mature virion (MV), previously known as the intracellular mature virion (IMV). In this review, the most recent virion nomenclature will be used. The MV is found in the host cell cytoplasm and is comprised of the viral core, lateral bodies, and a lipid membrane [48]. The MV particle, without any additional membranes, is only released during cell lysis. However, the MV can acquire a second membrane from the trans-Golgi network or endosomal vesicles and form the wrapped virion (WV), previously known as the intracellular enveloped virion (IEV) [51]. The WVs are transported on microtubules to the host cell surface, where they fuse with the plasma membrane [43]. Fusion of the WVs with the plasma membrane results in the generation of two types of extracellular virions (EVs): the cell-associated extracellular virion (CEV) or enveloped extracellular virion (EEV) [43]. The CEV and EEV forms are discernable from one another based on whether

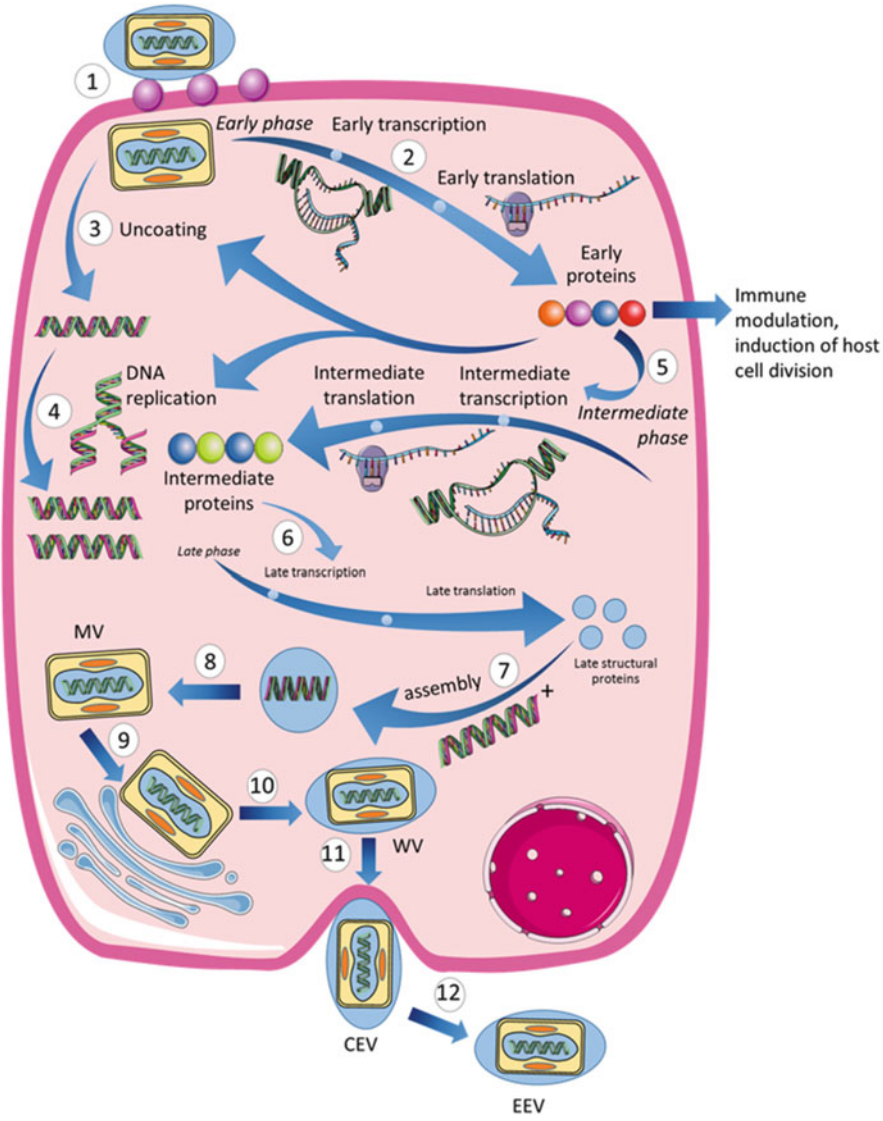

Fig. 3.1 The orthopoxvirus life cycle. *1* Poxvirus binds to host cell and enters via macropinocytosis. *2* Initial uncoating, early gene transcription, and translation by host ribosomes. *3* Early gene products aid in fully uncoating the virus to release genomic DNA into the cytoplasm. *4* DNA polymerase and other early gene products mediate replication of the genome in cytoplasmic replication factories. *5* Early proteins recognize intermediate gene promoters to initiate transcription followed by intermediate gene translation. *6* Intermediate gene products allow for late gene transcription and translation to proceed, producing structural proteins required for virion assembly. *7* Unit-sized genome and structural proteins unite, become wrapped in an intracellular host membrane, and assemble crescent-shaped virions. *8* Morphogenesis to generate the brick-shaped MV. *9* Some MV particles are trafficked to the Golgi body along microtubules. The remainder of the MV particles are released upon host cell lysis. *10* Formation of the WV following procurement

the particle remains attached to the host cell membrane (CEV) or is released extracellularly (EEV) [43].

Given the differential nature of the membranes, MVs and EVs have differences in their binding and entry mechanisms. MVs bind to glycosaminoglycans, among other host receptors. EVs, on the other hand, have no well-defined host cell binding targets [52]. There has been significant debate as to whether endocytosis or fusion is the primary mechanism of entry for poxviruses [52]. Recent data using live fluorescence microscopy imaging indicates that the primary mechanism is via endocytosis, specifically macropinocytosis [53–55]. Apoptotic mimicry via the phosphatidylserine in the viral membrane and epidermal growth factor receptor (EGFR) signaling have been implicated in MV-induced macropinocytosis [53, 56]. In contrast, EVs depend on acid-mediated disruption of their outer membrane, EGFR, and other signaling for macropinocytosis, without the need for phosphatidylserine [52, 56]. The EV-specific factors needed to induce macropinocytosis have yet to be elucidated [52]. Upon trafficking of the virion into the endocytic pathway, viral membrane fusion with the endocytic vesicle is thought to occur through a 12-protein entry/fusion complex [52]. Through an unknown molecular mechanism, the virion fuses with the endocytic membrane, and the viral core is released into the cytoplasm where it begins to transcribe early genes.

3.3.6 Life Cycle: Early Gene Expression

Core particles accrue around the nucleus, and following an initial incomplete uncoating event, the viral DNA-dependent RNA polymerase transcribes the early genes from the double-stranded DNA genome [44]. Early gene products include DNA-dependent DNA polymerase required for viral replication, viral transcription factors essential for intermediate gene expression, and secretory factors whose functions serve to enhance viral replication and prevent recognition by the host immune system [43]. Early gene products also include enzymes involved in complete uncoating of the poxvirus core, a step crucial to the replicative success of the virus [43]. Once the virus uncoats completely and releases genomic DNA into the cytoplasm, the early phase of the replicative cycle ceases, and viral genomic replication begins [43].

Fig. 3.1 (continued) of a secondary membrane from the trans-Golgi cisterna. *11* Transport to the host cell membrane is mediated via microtubules. The lipid bilayer of the virion fuses with the host plasma membrane. The CEV form remains attached to the surface of the host cell, infecting neighboring cells via modulation of host cell actin. *12* Generation of the EEV via exocytosis from the host cell. Adapted from [43]

3.3.7 Life Cycle: DNA Replication

In contrast to other DNA viruses, poxviruses replicate in the cytoplasm instead of the nucleus. Therefore, they must carry or encode all of the requisite machinery for DNA replication and transcription. This machinery includes viral enzymes such as DNA polymerase, helicase, ligase, and single-strand binding protein, among others. While not essential for replication, poxviruses also carry several conserved enzymes for metabolism of deoxyribonucleotides to augment replication in host cells with low endogenous levels of nucleotides [46].

Recent evidence indicates that the origins of replication for VACV are located near the genome termini and at junctions of concatemers [57]. Current data suggests that poxviruses replicate using a discontinuous or semi-discontinuous DNA replication strategy, which is consistent with nonviable VACV primase mutants [57, 58]. Upon completion of DNA synthesis, a head-to-head or tail-to-tail concatemer is cleaved by resolvase in order to resolve concatemers [46]. Resolution of concatemers generates progeny genomes that are ready to be packaged into the shell of a core particle [46].

3.3.8 Life Cycle: Intermediate and Late Gene Expression, Morphogenesis, and Dissemination

Generation of the components required to form the core particle relies on intermediate and late gene expression [46]. Intermediate gene products are often transcription factors and serve to recognize promoters for late gene transcription [59, 60]. The late class consists of virion structural proteins and early transcriptional machinery [41]. Recent temporal interrogations of the poxvirus transcriptome further detail these gene expression patterns [61, 62].

Approximately 6 h post-infection, electron microscopy shows the earliest discernable poxvirus structures depicted as a crescent-shaped immature virion [63]. This crescent-shaped particle will continue to undergo morphological changes and membrane procurement, forming the MV [63]. The majority of the MVs cease morphogenesis and are released upon cell lysis. A minority of the MVs are trafficked via microtubules and acquire membranes from endosomes or the trans-Golgi cisterna, forming the WVs [48]. The WVs then associate with microtubules once more to be trafficked to the cell membrane, where they form either CEVs or EEVs. The CEVs modulate actin into projections known as actin tails in order to spread to nearby cells. The EEVs exit via exocytosis. The mechanisms of cell exit have been well-detailed by Roberts and Smith [44].

The different virion morphologies correlate with modes of dissemination in and between hosts. Within the host, CEVs are thought to be responsible to cell-to-cell transmission, whereas systemic infection is mediated by infected leukocytes or EEVs [64]. For transmission between hosts, MVs play a central role because of their stability at room temperature and desiccation resistance [64].

3.4 Smallpox Clinical Illness

As the last naturally occurring case of smallpox was recorded in 1977, our understanding of the clinical course and manifestations of human smallpox have relied on historical clinical data. Thus, virologic or immunologic correlates underlying the clinical sequelae of smallpox remain unknown. Globally, smallpox was considered to be a uniformly severe disease with a high case fatality rate (CFR); however, cases of mild disease prior to the end of the nineteenth century challenged this assertion [6]. Mild smallpox-like disease with a CFR of ~1% in South Africa and North America was reported by Korté and Chapin, respectively, in the early twentieth century [6]. Thorough analyses of VARV samples gathered from various outbreaks have demonstrated that this mild form of smallpox was due to variola minor. Variola major, the primary causative agent of smallpox, had a CFR of 5–25% (or occasionally higher) [6], whereas the CFR of variola minor was approximately 1% [65]. Death from smallpox disease is generally attributed to immune complex-mediated toxemia, pneumonia, and hypotension, though debate exists surrounding the role of each of these processes [66, 67].

It has been suggested that genomic differences between variola major and minor may have limited contributions to the differential pathogenesis for these two strains as the genomes differ by only 2% and genes related to immunomodulatory functions within the host are unaffected [28, 68]. Thus, host-specific factors are likely related to the different CFRs. However, these remain elusive due to the limited availability of clinical samples from smallpox patients, the strict species tropism of VARV, the lack of an animal model of VARV infection that recapitulates human disease, and the restrictions on research investigations with VARV which have been in place since the eradication announcement in 1980.

3.4.1 Clinical Classification of Variola Major Infections

In 1972, the WHO adopted a clinical classification system for variola major infections proposed by Rao that categorized clinical type by the nature and evolution of the smallpox rash [69]. The WHO classifications are summarized below [6].

3.4.1.1 Ordinary Type

Ordinary-type smallpox was the most common clinical type of smallpox with an incubation period of 7–19 days (most commonly 10–14 days) [6]. The end of the incubation period was accompanied by the onset of fever (38.5–40.5 °C). Additional preeruptive stage symptoms also included (in order from highest to lowest frequency) generalized headaches, backaches, vomiting, and diarrhea. The frequency and severity of these symptoms were more typically associated with variola major infections than variola minor. Fever typically lasted 2–4 days during the prodromal period, fell at the onset of the macular rash appearance, rose again by days 7–9, and remained throughout the remaining disease course until scab formation on the pustular skin lesions. The smallpox rash demonstrated a centrifugal distribution

pattern and was typically densest on the face followed by the extremities. This pattern could be further subdivided with higher density on the distal portions of the extremities than proximal regions. Rash was usually (1) more profuse on the upper half of the face than the lower, (2) denser on the chest than abdomen, and (3) denser on the back of the trunk than the front.

Lesions first appeared on mucous membranes (including the tongue, palate, and pharynx) ~24 h prior to the appearance of macular rash. Skin lesions began on the face or forehead followed by proximal portions of the extremities, the trunk, and the distal extremities. However, lesion formation was very rapid (~24 h) with the order of their appearance seemingly indistinguishable. Although lesion sizes were highly variable, they were typically at the same stage of development within a specific body region. Thus, scab formation on the face could precede scabbing on distal regions of the body. Lesion formation could continue for an additional 1–2 days but was not typical outside of this period. Lesions were typically raised by the second day of macular rash due to fluid influx in the tissue spaces and were described as papules. Papules became vesicular (typically 2–4 days post-rash) with an opalescent fluid followed by a pustular stage in which the fluid became opaque/turbid (5–7 days post-rash). This shift in the turbidity was preceded by cellular necrosis and leukocyte infiltration followed by extensive tissue damage. The pustules reached their maximum size by 10 days post-rash. Pustule resolution largely began by the 11th day and was accompanied by flattening of the lesions, fluid reabsorption, hardening, and scab or crust formation (14–21 days post-rash). In contrast, lesions on the palms of the hands or soles of the feet typically persisted longer due to the thick stratum corneum. Moreover, they did not protrude from the surface of the skin and were often artificially removed.

Rao further proposed that ordinary-type smallpox could be subdivided based on the extent of the macular rash [69]. *Discrete ordinary-type smallpox* was the most common clinical subtype of ordinary-type smallpox in variola major infections (42% of all unvaccinated individuals). Here, skin lesions were discrete (separated by normal skin) and fewer in number than other subtypes. The CFR in Rao's case series was 9.3% [69]. *Confluent ordinary-type smallpox*, in which the pustular skin lesions were confluent on the face and surfaces of the extremities, was associated with fatal disease in 62% of unvaccinated individuals [69]. *Semiconfluent ordinary-type smallpox*, associated with a 37% CFR in unvaccinated individuals [69], was typified by a confluent pattern of skin lesions on the face but more disparate pattern on the rest of the body.

3.4.1.2 Modified Type

Modified-type smallpox, in which pustular skin lesions were less numerous than in ordinary-type smallpox, was primarily associated with vaccinated individuals and had an accelerated clinical course of disease. Scab/crust formation was usually complete within 10 days and disease was not fatal. Ricketts originally proposed that lesions were generally smaller and often of different conformations as compared to those found in ordinary-type smallpox [70]. However, subsequent reviews by Marsden suggested that classification of modified-type smallpox based on lesion

characteristics would be problematic as variola minor infections presented with similar lesion patterns [6, 71]. Modified-type smallpox was found in 25% of cases in vaccinated individuals but only 2% of unvaccinated cases [69].

3.4.1.3 Flat Type

Flat-type smallpox was only associated with variola major infections and had high CFRs in both unvaccinated and vaccinated patients (97% and 67%, respectively) [6]. Although quite rare (~7% of smallpox infections), the majority of cases were found within children (72%). Clinical symptoms associated with the prodromal period of ordinary-type smallpox were often more severe and continued following macular rash development. Skin lesions were flat in appearance and "soft and velvety" to the touch, contained little fluid, and did not follow the classic centrifugal distribution. However, lesions on the dorsal portions of the hands and feet sometimes displayed a pustular appearance. The central portion of flat-type smallpox lesions was often black or dark purple during illness and adopted an ash gray color 1–2 days prior to death. Respiratory complications often developed 7–9 days post-fever onset, and patients were febrile throughout the course of illness. Death typically occurred 8–12 days post-fever onset. It has been postulated that flat-type smallpox was associated with a deficient cellular immune response based on the appearance of the lesions. In those that survived flat-type smallpox, scabs typically formed 13–16 days post-fever onset and were thin and superficial.

3.4.1.4 Hemorrhagic Type

Hemorrhagic-type smallpox was rare (~3% of patients) and occurred primarily in adults. However, it was nearly 100% fatal irrespective of vaccination status with death normally occurring prior to macular rash development. Postmortem investigations suggested that hemorrhagic-type smallpox was characterized by a sustained high viremia, severe platelet depletion, and insufficient humoral immune response. This subtype was further divided into early and late hemorrhagic-type smallpox. *Early hemorrhagic-type smallpox* was characterized by hemorrhage (primarily subconjunctival) early in the disease course. Generalized erythema, petechiae, and ecchymosis appeared by day 2 post-fever onset followed by generalized "finely textured matted" lesions across the entire body (day 3). Lesions became purple in color by day 4, and death occurred suddenly by day 6 with patients in a conscious state. Death was likely due to both cardiac and pulmonary complications. Rao reported that early hemorrhagic-type smallpox was more common in women than men with higher incidences in pregnant versus nonpregnant females [69].

In contrast, hemorrhages occurred following the appearance of rash in *late hemorrhagic-type smallpox*. Lesions progressed quickly from macules to papules; however, post-papular maturation was very slow with hemorrhages sometimes occurring at the base of the developing lesions. Death usually occurred between 8 and 10 days post-fever onset. *Late hemorrhagic-type smallpox* patients that presented with raised pustular lesions had a lower fatality rate than those with flat lesions. Hemorrhage was common in various mucous membranes albeit less

frequently than in *early hemorrhagic-type smallpox*. If lesions matured into pustules, hemorrhage was relegated solely to mucous membranes. In contrast to *early hemorrhagic-type smallpox*, the frequency of *late hemorrhagic-type smallpox* did not differ based on gender although pregnant women were found to be slightly more susceptible.

3.4.2 Clinical Classification of Variola Minor Infections

The clinical course of variola minor infection has been defined primarily by Marsden's observations of ~14,000 cases from 1928 to 1934 [72] and supplemented by additional observations [73–75]. Clinically, the disease symptoms of variola minor mimic those found in mild variola major infections albeit far less severe. Fever onset was sudden and was accompanied by severe headache and backache. Vomiting may also be found during this period. Secondary fever was extremely rare. Patients often remained ambulatory throughout the disease course, and the individual skin lesions were often smaller in size than those associated with variola major. The sequence of lesion formation and overall distribution patterns were also similar to variola major; however, the evolution of the lesions from papules to pustules and scab formation was far more rapid. Papules often became vesicular within 3 days and pustular by 4 days following initial papule formation. Crust/scab formation normally began by days 6–7. Although determination of CFRs from larger cohorts of patients could be used to distinguish between outbreaks of variola major or minor, individual cases of variola minor infection were impossible to discern from either discrete ordinary- or modified-type smallpox. Hemorrhagic-type smallpox was noted in Marsden's cohort of variola minor infections; however, these comprised 0.02% of all cases [72]. MacCallum and Moody noted that pregnant women within their patient cohort were vulnerable to hemorrhage [6, 74].

3.5 Smallpox Pathogenesis

Although informative, smallpox pathogenesis investigations in humans have been largely limited to samples acquired during clinical illness, convalescence, or post-mortem. As over 40 years has passed since the last natural case of smallpox, many of these analyses were technologically limited by today's standards. Further, hypothesis-driven investigations of in vitro or in vivo VARV infections have also been limited due to the strict constraints placed on research involving live VARV.

3.5.1 Viral Entry and Infection

Epidemiological analyses suggest that the primary route of VARV infection was through the inhalation of mucous excretions from the nose or mouth of an infected patient resulting in the deposition of virus in the respiratory tract [6, 76,

77]. Historical analyses have demonstrated that VARV was not shed in mucous discharges from infected patients until the end of the incubation period when enanthem appeared. To shed light on this, various groups have employed respiratory models of *orthopoxvirus* infections in vivo using various animal species. Aerosol infection of mice with mousepox demonstrated that upper and lower respiratory tract mucosal cells and alveolar macrophages were the primary targets of viral infection [78]. More recently, Johnson and colleagues have investigated CPXV pathogenesis in rhesus macaques following respiratory inoculation. Intrabronchial (*i.b.*) administration of 5×10^5 plaque-forming units (pfu) of virus resulted in systemic disease. In contrast, administration of CPXV by small- or large-particle aerosol droplets resulted in limited systemic dissemination of virus [79, 80]. Aerosol delivery of high doses of MPXV, which presents with similar clinical symptoms as VARV in humans, to cynomolgus macaques resulted in similar observations as those seen with aerosolized CPXV: lethal disease with inconsistent lesion development [81, 82].

In contrast, cutaneous infections through accidental inoculation or variolation largely resulted in mild disease with CFRs of 1–2% [68]. VARV infection through the conjunctiva remains unclear. Although variolous conjunctivitis was confirmed by Kempe, it is unknown whether this was a true portal of viral entry [83]. Congenital infection is equally unclear. Rao noted congenital smallpox in 10 babies born from mothers that had variola major infections out of 113 babies that survived delivery (35% of the total pregnancies in this cohort were abortive or stillborn pregnancies) [69]. Congenital infection during pregnancy in variola minor-infected mothers was more informative. An analysis by Marsden and Greenfield reported that 50% of babies born to mothers with variola minor infections did not acquire in utero infection [84]. Babies who contracted variola minor in utero experienced a delayed disease course, and it was presumed that the fetus was infected following viral replication in the placenta [6].

3.5.2 Viral Dissemination

Upon deposition of virus in the respiratory tract, VARV is thought to migrate to nearby lymph nodes and replicate in the reticuloendothelial system. Subsequent invasion of the dermis and continued replication in mucous membranes result in smallpox lesion development [66]. While data for other poxviruses is available, there is a paucity of information regarding viremia during the smallpox disease course in humans [66]. As compared to current routinely employed diagnostic methods for assessing pathogen presence or loads, viremia in smallpox patients was primarily determined with the chick chorioallantoic membrane assay. The development of viremia was related to the smallpox clinical type. Virus was rarely recovered from whole blood or serum in ordinary-type smallpox [85–87]. In contrast, high titer virus was recovered throughout the course of illness in hemorrhagic-type smallpox, and viral loads were consistently higher in early hemorrhagic-type smallpox as compared to the late hemorrhagic type [86–89].

Oral and pharyngeal samples from infected patients demonstrated high amounts of virus with peak titers at days 3–4 of disease and that viral titer and viral persistence had a positive correlation with disease severity [90]. The high viral load generally persisted for 7–13 days post-fever onset in nonfatal disease and up to death in fatal cases [90]. Investigations of orthopoxviruses in animal models suggest that they are disseminated throughout the host by infected leukocytes or, to a lesser extent, as free virions [6, 68]. Jahrling and colleagues demonstrated that VARV dissemination in cynomolgus macaques was primarily achieved by infected monocytes and macrophages [91].

3.5.3 Clinical and Anatomic Pathology

Recently, Cann et al. provided a concise review of the historical pathological evaluations of smallpox [92]. We will summarize these here, but the reader is recommended to access this review for more detailed descriptions. Clinical pathology data from patients with ordinary- or hemorrhagic-type smallpox demonstrated leukocytosis, thrombocytopenia, and coagulation abnormalities [93–96]. Mild anemia was found in ordinary-type smallpox patients but was absent in those with hemorrhagic-type smallpox [93, 94, 97]. Thrombocytopenia would also often resolve during the pustular stage in ordinary-type smallpox but did not resolve in hemorrhagic-type smallpox cases prior to death [94–99].

Historically, postmortem pathological examination of fatal smallpox illness largely focused on skin and mucosal lesions. Pathological changes in other major organ systems were poorly described or unreported. Mucosal lesions were similar to those found on the skin with the exception that ulceration was common in mucosal lesions and healing occurred in the absence of scab formation [92]. Non-specific splenic changes were common in both ordinary-type and hemorrhagic-type smallpox with sizes ranging from normal to mildly enlarged. Macrophage-rich sinusoids were often described. Focal areas of necrosis were also described with large amounts of bacteria. Lymph nodes were often described as normal, but hypertrophy and hyperemia were also reported. Debate over the contribution of viral interstitial pneumonitis versus bronchopneumonia from a secondary bacterial infection to lung pathology exists. Regardless, lung pathologies in ordinary-type smallpox were often related to death [67, 92]. Edema and atelectasis were also commonly reported. Lung pathology in hemorrhagic-type smallpox resembled ordinary-type smallpox with the exception of multifocal acute hemorrhages. Mild to marked hepatomegaly was the most common pathologic change noted in the liver from both fatal ordinary-type and hemorrhagic-type smallpox. Multifocal necrotizing hepatitis was also commonly described. Myeloid hyperplasia predominated in the bone marrow of ordinary-type smallpox cases, whereas hematopoietic necrosis was found in hemorrhagic-type smallpox. Polymorphonuclear cell reduction or absence was highly reported in the absence of information regarding the specific smallpox classification type. Testicular lesions, primarily multifocal interstitial orchitis, were common and did not differ based on the disease type. Ovarian lesions were less common than testicular lesions

but were similar in character. Kidneys were grossly normal with tubulointerstitial nephritis in ordinary-type smallpox, but extensive pelvic and ureteral hemorrhage was noted in hemorrhagic-type smallpox. Results from pathological analysis of the central nervous system were rarely reported, and cardiac pathology due to VARV infection appeared to be rare.

3.5.4 Smallpox Pathogenesis in Animals

Humans are the only known natural or permissive hosts for VARV. However, concerted efforts have been made to develop a VARV infection model in animals that recapitulates human disease. Although there have been relatively few published reports following the announcement of VARV eradication (primarily in nonhuman primates), there were considerable efforts to identify animal species that were susceptible to VARV infection in the early and mid-twentieth century. Interestingly, many of these reports had assumed that VARV could be transformed into VACV by multiple passages of virus through animals. Although this postulate was ultimately rejected following scientific advancements in the mid- to late twentieth century, these investigations provide important information about the range of different animal species that were examined for VARV susceptibility. A comparison of animal species, inoculation methods, virus species/strain, and disease course are presented in Table 3.1.

Herrlich et al. published an exhaustive investigation of VARV susceptibilities within various mammalian species [100]. Previous investigations of VARV infections in rabbits had produced confounding results in regard to symptomatic infections [120–123]. Although there was evidence that local lesion development could be achieved following infection of animals with VARV that had undergone successive passaging in calves or rabbits, this procedure was not universally successful. Herrlich and colleagues generated stocks of VARV following successive passages in various systems including eggs, cell culture (HeLa cells, porcine kidney cells, bovine kidney cells, and embryonic muscle cells), and infant mice. Intraperitoneal (*i.p.*) inoculation of infant mice with mouse-passaged VARV resulted in a lethal phenotype with high viral titers in the lungs, liver, kidneys, and spleen. Intracerebral (*i.c.*) inoculation of infant mice with the same material resulted in both high lethality and high viral titers within the brain and internal organs. Subcutaneous (*s.c.*) inoculation resulted in asymptomatic disease although viral amplification was noted within the lungs. Nonhuman primate infections (cutaneous (*c.*), *s.c.*, and intravenous (*i.v.*) administrations) were performed with non-passaged VARV. Disease severity ranged from mild to lethal, and rash development was noted in all infected animals. Infant rabbits did not show any signs of infection following dermal (*d.*), *i.p.*, or *i.n.* administrations of VARV. Subcutaneous administration of high titers of VARV resulted in the recovery of limited amounts of infectious virus. Adult rabbits were equally poor surrogates for VARV infection. Dermal, intravenous, and intracerebral inoculations of high titers of VARV resulted in asymptomatic disease. Intradermal (*i.d.*) inoculations resulted in early post-infection development of highly

Table 3.1 VARV pathogenesis in small and large animals

Host		Route of inoculation	Strain	Inoculation dosage	Clinical presentation	Clinical severity	References
Infant mice		i.p.	Bombay	100–100 egg-infective virus units (EIV)	Not described	Severe disease	[100]
		i.c.	Bombay	n.d.	Not described	Severe disease	[100]
		s.c.	Bombay	n.d.	Asymptomatic	No disease	[100]
SCID mice		i.n.	Ind-3a	5.2 $\log_{10}$ pfu	Asymptomatic	No disease	[101, 102]
Nonhuman primates	Cynomolgus macaque	Scarification (s.), corneal (c.), mucous membranes (m.m.), s.c., i.c., i.b., i.t., i.n.	n.d. (variola major)	n.d.	Localized lesions at inoculation site. Development of fever in some cases. Reports of generalized exanthema	Mild disease	[103–106]
	Rhesus macaque	s.c., i.n., i.d., i.p., i.b., oral (o.)	n.d. (variola major)	n.d.	Localized lesions at inoculation site. Generalized exanthema and fever reported	Mild disease	[106–108]
	Pig-tailed macaque	s., i.t., o., i.v.	n.d. (variola major)	n.d.	Localized lesions at inoculation site. Generalized exanthema and fever reported	Mild disease	[106, 109, 110]
	Toque macaque	i.t.t.	n.d. (variola major)	n.d.	Development of orchitis accompanied by fever and generalized exanthema	Mild disease	[108, 111]
	Green monkey	i.t.t., s.	n.d. (variola major)	n.d.	Development of orchitis accompanied by fever and generalized exanthema	Mild disease	[112]
	Common squirrel monkey	i.b.	n.d. (variola major)	n.d.	Fever and generalized exanthema	Mild disease	[106]

	Patas monkey	s.	n.d. (variola major)	n.d.	Development of local lesions	Mild disease	[112]
	Cynomolgus macaque	s.	n.d. (variola minor)	n.d.	Development of local lesions	Mild disease	[113]
	Rhesus macaque	i.v., s.c.	n.d. (variola minor)	n.d.	Local lesions and fever	Mild disease	[113–115]
	Toque macaque	s.c.	n.d. (variola minor)	n.d.	Asymptomatic	No disease	[116]
	Cynomolgus macaques	Aerosol	Yamada	5×10^4 infectious units	Fever and general malaise. Generalized exanthema	Mild disease	[117]
	Cynomolgus macaques	cut.	Bombay	n.d.	Primary pustules	Mild disease	[100]
	Rhesus macaques						
	Cynomolgus macaques	i.v.	Harper	10^8 pfu	Peripheral lymphadenopathy (day 5 p.i.), exanthema, and enanthema (day 5 p.i.) with numerous macules and papules on legs, arms, face, lips, and tongue	Ordinary-type smallpox. Low mortality	[118]
		i.v.	Harper	10^9 pfu	Accelerated disease course. Secondary systemic bacterial infections (day 4 p.i.). Petechial rash. No papules or pustules. Lymphadenopathy (day 3 p.i.)	Hemorrhagic-type smallpox. Universal lethality	[118]

(continued)

Table 3.1 (continued)

Host	Route of inoculation	Strain	Inoculation dosage	Clinical presentation	Clinical severity	References
Prairie dog	i.n.	Solomain	6.6×10^6 pfu	Slight redness at nares. No lesions observed	No disease	[119]
	i.d.	Solomain	6.6×10^6 pfu	Slight erythema at inoculation site. No lesions observed	No disease	[119]

erythematous skin lesions; however, these resolved by days 3–4 post-infection. Corneal administration of VARV resulted in the generation of low amounts of infectious virus. Intratesticular (*i.t.t.*) inoculation resulted in transient topical symptoms that were not described in detail. Scarification of pigs with non-passaged VARV resulted in asymptomatic disease and a transient mild exanthem on day 5 post-infection that resolved on days 6–7. Administration of VARV by various routes to calves, a single sheep, and goat resulted in asymptomatic disease. Recently, Carroll et al. investigated VARV pathogenesis in the North American black-tailed prairie dog [119]. This followed the demonstration that MPXV infection in this animal species resulted in a similar clinical course of disease to humans [124, 125]. Although *i.n.* and *i.d.* administration of VARV resulted in seroconversion within the infected animals, there were no clinical signs of disease [119].

Prior to Herrlich's investigation, Hahon summarized the available literature regarding experimental infection of nonhuman primates with VARV and the contributions of virus species, strain, route of inoculation, and host species to disease severity [126]. These are summarized below and data is presented in Table 3.1. In general, experimental infection of nonhuman primates with VARV (major or minor) by almost any route of inoculation resulted in mild infection that resolved in ~14 days. Dermal or *i.d.* inoculation resulted in the development of a local lesion at the site of inoculation, followed by fever on ~day 8 post-infection and a generalized exanthema by days 9–10. Infection of nonhuman primates by respiratory inoculation largely mimicked *d.* and *i.d.* inoculation; however, local lesion development did not occur. Administration of VARV to mucous membranes resulted in generalized exanthem in only ~10% of infected animals (as compared to 70–80% of *d.* or *i.d.* inoculated animals) in the absence of general malaise or abrupt fever. Infection of nonhuman primates with variola minor resulted in a similar pattern of disease as variola major infection. Although there was a scarcity of data published regarding the relation of clinical illness with nonhuman primate species, Hahon's unpublished data suggested the cynomolgus macaques had a greater occurrence of generalized exanthem as compared to rhesus macaques [126]. Magrath and colleagues performed a comprehensive study comparing the virulence of different VARV strains [127]. Here, the authors suggested that clinical disease in nonhuman primates was related to the severity of clinical disease in humans for a particular virus strain. For example, infection with variola major acquired from a case of confluent-type smallpox in humans resulted in more severe disease in nonhuman primates than variola minor.

More recently, Jahrling and colleagues performed a detailed investigation of VARV pathogenesis in cynomolgus macaques comparing viral strain (Harper vs. -India), dose (10^6–10^9 pfu), and route of inoculation (*i.v.* vs. aerosol) [91]. Detailed outcomes from this investigation cannot be made due to discrepancies in disease outcome between the data presented for individual animals and that presented for each experimental cohort. However, a subsequent investigation by Wahl-Jensen and colleagues detailed the temporal progression of VARV pathogenesis in cynomolgus macaques [118]. Intravenous inoculation of 10^8 pfu VARV (Harper strain) resulted in significant lesions by day 3 post-infection, exanthema and enanthema by day

5, and peak cutaneous lesions by days 7–8. Lymphadenopathy was noted in all animals by day 5. In contrast, *i.v.* inoculation with 10^9 pfu resulted in an accelerated disease course resembling hemorrhagic-type smallpox with all animals being euthanized by day 4 post-infection. Cutaneous lesions were limited to a petechial rash in most animals by day 3. Lymphadenopathy was noted in all animals by day 3. Three animals developed a secondary bacterial infection.

In the future, humanized or immune-modified mouse models may play a role in establishing animal models for human smallpox disease [101, 102]. Other orthopoxvirus animal models may also contribute, though they are limited in their ability to recapitulate human disease. In the absence of an adequate animal model, serological and/or in vitro studies with VARV at WHO-approved collaborating centers may contribute to efficacy assessment of new prophylactics and therapeutics.

3.6 Diagnosis

3.6.1 Clinical Diagnosis

The early maculopapular rash in smallpox disease can resemble that of many other viral, bacterial, or iatrogenic illnesses, which are reviewed in [66]. Upon the appearance of papulovesicular lesions, chickenpox is often confused with smallpox. However, the increased severity of constitutional symptoms and centrifugal rash distribution in smallpox distinguishes the disease from the less severe presentation and centripetal rash distribution in chickenpox, among other features as described in [128]. Recognition of potential smallpox disease early in its course is key to prevent outbreaks. To facilitate this, the CDC has provided an evaluation algorithm for clinical evaluation of a patient with potential smallpox [129].

3.6.2 Laboratory Diagnosis

In the event of identification of a high-risk patient using the CDC guidelines, specimens should be collected and shipped to a qualified laboratory per the guidelines on the CDC website, and public health authorities should be notified immediately [130]. Isolation of the patient, contact tracing, and vaccination of contacts should then ensue [128].

Multiple laboratory techniques exist for orthopoxvirus identification. Light microscopic examination of histologically stained tissue samples reveals characteristic Guarnieri bodies [131]. Additionally, the distinct oval to brick morphology of virions can be identified by electron microscopy; however, these microscopic techniques cannot distinguish between orthopoxviruses of different species. Immunological and virological techniques have also been utilized in diagnosis. Viral outgrowth can be performed in several cell culture models and on chorioallantoic membranes of embryonated chicken eggs, and VARV can be distinguished from other orthopoxviruses based upon restricted growth above 39 °C [132–134]. Enzyme-linked

immunosorbent assays, immunofluorescence microscopy, and plaque reduction neutralization tests have also been developed for detection of orthopoxvirus serologies. However, all of these techniques are limited in their ability to distinguish between orthopoxviruses of different species [135].

The most sensitive and specific diagnostic technique for orthopoxviruses are nucleic acid-based molecular tests. Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis can distinguish between orthopoxviruses based on the amplicon or restriction fragment pattern, respectively [134, 136]. Oligonucleotide microarrays have also been employed to differentiate between orthopoxviruses [137]. Notably, the advent of quantitative PCR assays has supplanted traditional PCR or RFLP analyses because of its extremely high sensitivity, low cost, and speed [138, 139]. The state of the art for quantitative PCR assays for VARV is described in [138].

3.7 Prophylaxis

After Jenner's development and validation of smallpox vaccination, containment and reduction in smallpox disease prevalence were slow. Early in the nineteenth century, a manufacturing method was developed to produce vaccine on the skin of calves; however, this vaccine was not heat-stable and decayed within days [140]. The logistical challenges of distributing an unstable vaccine drastically impeded progress against smallpox. Indeed, over 300 million deaths occurred in the twentieth century alone despite the ability to produce an effective vaccine [140]. Optimization of vaccine production methods in the 1950s resulted in the ability to produce large amounts of heat-stable vaccine. As the SEP began in the 1960s, the WHO established a standard production protocol to ensure stable and reliable vaccine stocks. These vaccines were the first-generation smallpox vaccines and were employed pervasively throughout the world during the SEP [140]. Their usage resulted in the eradication of smallpox, with the last known transmitted case occurring in 1977 after years of ring vaccination efforts from dedicated volunteers throughout the world [6].

The potential for smallpox or smallpox-like disease to emerge in human populations in tandem with modern-day bioterrorism events such as the 2001 dissemination of *Bacillus anthracis* through the mail has provided impetus to stockpile smallpox vaccines [15, 141]. Moreover, further research into the efficacy of current vaccines and therapeutics against other orthopoxviruses is warranted. Pre-event vaccination has been considered; however, much of the global stockpile of vaccine is associated with significant adverse events as well as multiple contraindications [15, 141]. New generation vaccines may overcome these issues, but several issues regarding vaccine safety, efficacy, and supply need to be addressed [142, 143].

In this section, we present an overview of first-, second-, and third-generation vaccines in clinical development or with regulatory approval as of 2017, as well as fourth-generation vaccines in preclinical development. We review the current

knowledge regarding the safety and efficacy of the vaccines within each generation. Preclinical data for the vaccines has been reviewed in detail by Meyer [144]. Vaccine efficacy is considered in light of the gold standard for smallpox vaccine success: the incidence of a primary vesicle 6–8 days after multi-puncture administration with a bifurcated needle, also known as vaccine take [145].

3.7.1 First-Generation Vaccines

First-generation vaccines are composed of VACV derived from animal scarification, usually from the skin of calves. Their major limitation is the high risk of adverse events, attributed to the use of replication-competent VACV and contaminating adventitious agents acquired during the manufacturing process. Indeed, depending on the VACV strain used in the vaccine, these vaccines were associated with mortality rates of 1.4 to 8.4 per million primary vaccines during the pre-eradication era [146]. Conversely, these vaccines have proven endemic efficacy, as they were used during the SEP and were successful in eradicating the disease. Moreover, these vaccines can reduce the severity of disease post-exposure [6].

Numerous strains of VACV were used in first-generation vaccines; however, controlled trials for most strains were either not conducted or conducted in the post-eradication era. For example, vaccination in the United States was discontinued in the 1970s, except for military personnel who were vaccinated until 1989 because of concerns over bioterrorism use during the Cold War. In addition, after the anthrax bioterrorism attacks in 2001, vaccination of hospital and at-risk military personnel occurred [147, 148]. These vaccination programs provide an important source of data for rigorous evaluation of first-generation vaccine safety. Here, the most commonly used strains for which clinical data regarding efficacy and safety is available are presented in Table 3.2. Notably, most of the vaccines used during the SEP were derived from the New York City Board of Health (NYCBOH) and Lister strains. Other strains used in eradication efforts are detailed in [6].

Vaccination with first-generation vaccines is highly successful and protective against smallpox disease. Epidemiological evidence exists that demonstrates some protection against smallpox disease or death up to 20 years after vaccination [159]. Both cell-mediated and humoral immunities are thought to be involved in protection against smallpox disease [160]. Evidence from a macaque model indicates that the humoral response to the Dryvax smallpox vaccine is necessary and sufficient for protection from MPXV [161]. Importantly, stable antibody responses up to 75 years after vaccination have been demonstrated in humans, with variable stability of antiviral T-cell responses [162]. These data must be tempered, however, with the understanding that definitive human immunological correlates of protection for smallpox are unattainable because of the disease eradication and obvious ethical issues for human studies. The limited ability of animal models to accurately reproduce human smallpox disease and immunological response has been reviewed by Moss [160] and addressed elsewhere in this chapter.

Table 3.2 Overview of first-generation smallpox vaccines

Major limitation	Rationale for use	Platform	Source strain	Vaccine	% Vaccine take (naïve)	% Vaccine take (previously vaccinated)	Production method	Regulatory status
1.4 [NYCBOH] and 8.4 [Lister] deaths per million primary vaccinees [146] Risk of adventitious infectious agent contamination	Proven endemic efficacy during the eradication of smallpox [6]	VACV derived from animal scarification	NYCBOH	Dryvax (Wyeth)	>95% [149, 150]	No clinical data available	Calf lymph	No longer licensed in the United States [151] Stockpiles exist in many countries [141] Policy in place for emergency use [142]
				APSV (Aventis Pasteur, now Sanofi Pasteur)	>95% [152]	No clinical data available	Calf lymph	Part of US national stockpile, investigational [152, 153] Stockpiles exist in many countries [141] Policy in place for emergency use [142]

(continued)

Table 3.2 (continued)

Major limitation	Rationale for use	Platform	Source strain	Vaccine	% Vaccine take (naïve)	% Vaccine take (previously vaccinated)	Production method	Regulatory status
			Lister	Lancy-Vaxina (Berna Biotech)	>95% [154–156]	>95% [155]	Calf lymph	Stockpiles exist in many countries [141] Policy in place for emergency use [142]
				Pourquier (Institut Vaccinal Pourquier)	No clinical data available	>95%% [157]	Calf lymph	
				Lister (Israeli Ministry of Health)	No clinical data available	61% [158]	Chorioallantoic membranes of chicken embryos	

Here, an overview of the most commonly used strains for which clinical data regarding efficacy and safety is available are presented. Notably, most of the vaccines used during the SEP were derived from the New York City Board of Health (NYCBOH) and Lister strains. Other strains used in eradication efforts are detailed in [6]

Table 3.3 Overview of second-generation smallpox vaccines in clinical development or with regulatory approval as of 2017

Major limitation	Rationale for use	Platform	Source strain	Vaccine	% Vaccine take (naïve)	% Vaccine take (previously vaccinated)	Production method	Regulatory status
High risk of serious adverse events from the use of virulent/ replication-competent VACV	Decreased risk of adventitious infectious agent contamination Expected endemic efficacy because source strains are nearly identical to first-generation vaccines	VACV derived from tissue culture	NYCBOH	ACAM1000 (Acambis/Sanofi Pasteur)	>95% [167–169]	No clinical data available	Lung fibroblasts (MRC-5)	See ACAM2000
			NYCBOH	ACAM2000 (Acambis/Sanofi Pasteur)	>95% [132, 168–170]	84–88% [170]	Lung fibroblasts (MRC-5)/ kidney epithelial cells (VERO)	Licensure (2007) in the United States for those at high risk for smallpox infection [153] Part of US national stockpile [153] Recommended for WHO vaccine stockpile [142]
			NYCBOH	CJ-50300 (CJ CheilJedang Corporation, South Korea)	>95% [171, 172]	>95% [173]	Lung fibroblasts (MRC-5)	Unknown regulatory status [144]
			NYCBOH	CCSV (DynPort)	>95% [174]	>95% [174]	Lung fibroblasts (MRC-5)	Investigational

(continued)

Table 3.3 (continued)

Major limitation	Rationale for use	Platform	Source strain	Vaccine	% Vaccine take (naïve)	% Vaccine take (previously vaccinated)	Production method	Regulatory status
			Lister	Elstree-RIVM (Netherlands)	>95% [175]	71–74% [175]	Primary rabbit kidney cells	Stockpiles exist in many countries despite lack of licensure [144] Policy in place for emergency use [142]
				Elstree-BN (Bavarian Nordic)	>95% [176]	No clinical data available	Chicken embryonic fibroblasts	

Table 3.4 Overview of third-generation smallpox vaccines in clinical development or with regulatory approval as of 2017

Major limitation	Rationale for use	Platform	Source strain	Vaccine	% Vaccine take (naïve)	% Vaccine take (previously vaccinated)	Production methods	Regulatory status
No proven endemic efficacy with significant alterations (attenuation, replication deficiency) to source strains	Improved safety profiles, especially in groups at high risk for complications	Attenuated VACV, replication competent	Lister	LC16m8 (Kaketsuken and VaxGen)	>95% [176, 178–180]	86.6% [178]	Primary rabbit kidney cells [176]	Licensed and stockpiled in Japan [141] Recommended for WHO vaccine stockpile [142]
		Attenuated VACV, replication deficient	Ankara	IMVAMUNE/ MVA-BN (Bavarian Nordic)	No vesicle formation	No vesicle formation	Chicken embryonic fibroblasts [181] BHK-21 [182] Avian suspension lines [183] EB66 [184]	Licensure in Europe and Canada [185] Part of US national stockpile, investigational [150, 153, 186–189] Policy in place for emergency use [144]
			Ankara	ACAM3000 (Acambis/ Sanofi Pasteur)	No vesicle formation	No vesicle formation		Investigational [190, 191]

(continued)

Table 3.4 (continued)

Major limitation	Rationale for use	Platform	Source strain	Vaccine	% Vaccine take (naïve)	% Vaccine take (previously vaccinated)	Production methods	Regulatory status
			Ankara	MVA (Therion Biologics)	No vesicle formation	No vesicle formation		Investigational [192]
			Copenhagen	NYVAC (Sanofi Pasteur)	No vesicle formation	No vesicle formation	Lung fibroblasts (MRC-5)/ kidney epithelial cells (VERO) [193]	Investigational [194]

Generally, vaccination with first-generation vaccines is unremarkable. The vaccine is administered by drawing reconstituted VACV between the two prongs of a bifurcated needle by capillary action and inoculating with multiple jabs over the deltoid muscle to produce visible bleeding. In naïve individuals, a vesicle forms approximately 6 days after vaccination, demonstrating a take and successful vaccination. The vesicle then becomes pustular, less swollen, and forms a scab which falls off in 2–3 weeks. For individuals who have been vaccinated previously, success is defined as the presence of a vesicle or an area of induration surrounding the vaccination site 6–8 days after vaccination. Notably, the presence of a papule/redness within 24 h and subsequent appearance of a vesicular lesion (within 3 days) are indicative of an allergic response to viral proteins and are not considered a major take or vaccine success [6].

First-generation vaccination is associated with minor adverse events including regional lymphadenopathy and constitutional symptoms [163]. Severe adverse events associated with first-generation vaccines include inadvertent inoculation, generalized vaccinia, eczema vaccinatum, progressive vaccinia, postvaccinial central nervous system disease, and fetal vaccinia [163]. More recently, in a Department of Defense and CDC vaccination program, vaccine-associated cases of myocarditis and ischemic cardiac events were reported [164, 165]. Management of these adverse events and contraindications for smallpox vaccination in a pre-outbreak setting are reviewed in [163, 166]. Notably, the use of first-generation vaccines for individuals at high-risk for smallpox exposure has no absolute contraindications. Relative contraindications must be balanced with the risk of exposure and subsequent smallpox disease.

3.7.2 Second-Generation Vaccines

Second-generation smallpox vaccines are defined by production in tissue culture from VACV strains very similar or identical to those used to produce the first-generation vaccines. These vaccines confer additional safety, as they have a decreased risk of bacterial or other adventitious contamination from the production process. Moreover, they are expected to have similar protective efficacy, because they are derived from source strains nearly identical to the first-generation vaccines that have proven field efficacy.

Second-generation vaccines are derived from either the NYCBOH or Lister strains and have comparable take rates to the first-generation vaccines (Table 3.3). ACAM2000®, a second-generation vaccine produced by Acambis (now Sanofi Pasteur), received Food and Drug Administration (FDA) approval in the United States in 2007 for those at high risk for smallpox infection. ACAM2000® was approved on the basis of six clinical trials demonstrating non-inferiority (take rate in naïve subjects and neutralizing antibody response in vaccine-experienced individuals) to the first-generation Dryvax vaccine, which are reviewed in [177]. Despite these encouraging results, ACAM2000®, when diluted, results in a decreased take rate. In contrast, Dryvax had higher take rates in vaccine-experienced

Table 3.5 Overview of fourth-generation (subunit) smallpox vaccines in preclinical development as of 2017

Platform	Vaccine gene (s)/protein(s)	Animal models tested	Protection	References
Protein	H3	Mice	80% from VACV	[197]
Protein	A33/B5/L1	Mice	Multiple protein combinations/ challenges tested	[198]
Protein	A27/A33/B5/ L1 ± D8	Mice	With D8, 66% from VACV Without D8, 26% from VACV	[199]
Protein	B5	Mice	100% from VACV	[200]
Protein	A27/A33/B5/ L1	Cynomolgus macaques	Variable protection from MPXV, depending on adjuvant used	[201]
Protein	A30/B7/F8	Mice	100% from VACV	[202]
Protein + DNA	A27L/A33R/ B5R/L1R	Rhesus macaques	100% from MPXV	[203]
DNA	A27L/A33R/ B5R/L1R	Mice, Rhesus macaques	Protection from MPXV in multiple studies	[204–207]
DNA	A30L/B7R/ F8L	Mice	80% from VACV	[202]
DNA	A27L/A33R/ B5R	Mice	B5R >100% from VACV A27L, A33R >66% from VACV	[208]

subjects and can also be diluted without compromising efficacy [149]. Regarding safety, as second-generation vaccines consist of replication-competent VACV, the risk of the adverse events described previously for the first-generation vaccines still exists. There was no difference in the number of adverse cardiac events (myopericarditis) observed between ACAM2000® and Dryvax [177]. Overall, the safety profile of second-generation vaccines is improved because of the decreased risk of adventitious contamination. Still, no safety data has been presented for ACAM2000® in children [177].

Additional second-generation vaccines that have entered clinical studies include the NYCBOH-derived CJ-50300 (from South Korea) and cell-cultured smallpox vaccine (CCSV) (from DynPort). CJ-50300 has a take rate >95% in both naïve and vaccine-experienced individuals with minimal reported serious adverse events [171–173]. CCSV also had a >95% take rate with no difference in adverse events when compared to Dryvax [174]. However, CCSV development was discontinued due to funding discontinuation from the Department of Defense [176]. The Lister-derived Elstree-RIVM (from the Netherlands) and Elstree-BN (from Bavarian Nordic) vaccines also show take rates >95%. The RIVM vaccine was field-tested in thousands of children in the 1970s in Indonesia and showed similar take rates and adverse events to that of calf-lymph vaccine [175]. The Elstree-BN vaccine was evaluated in a small phase I study in 2004 with similar results, but has not been published [176].

3.7.3 Third-Generation Vaccines

Third-generation vaccines are defined by the attenuation and/or replication insufficiency of VACV and are also produced in tissue culture. These vaccines theoretically confer a great safety advantage, as adverse events associated with the use of virulent, replication-competent VACV should be minimized. Conversely, these vaccines have no proven endemic efficacy and, unlike second-generation vaccines, have significant alterations to the source strains used in the SEP. Notably, because most third-generation vaccines in development are not replication competent, no vesicle formation is observed upon inoculation in clinical trials. Thus, immunological measures such as neutralizing antibodies and T-cell responses must be used as surrogates to measure vaccine success rates.

The only attenuated, replication-competent third-generation vaccine in clinical development is the Lister-derived LC16m8 vaccine (Table 3.4). The vaccine take rate ranged from 94.4 to 100% in naïve individuals and was 86.6% in previously vaccinated individuals [178]. Comparison of LC16m8 with Dryvax demonstrated non-inferiority in safety and immunogenicity [179]. The safety profile of LC16m8 is further supported by its use in thousands of children during the 1970s [180] followed by its licensure in 1980 in Japan.

Several attenuated, replication-deficient vaccines are in clinical development. Most of these are derived from the parent Ankara VACV strain and have undergone many serial passages in tissue culture to produce the modified VACV Ankara (MVA) strain [195, 196]. ACAM3000 is an MVA-based vaccine in development by Acambis (now Sanofi Pasteur), the producer of the second-generation ACAM2000. Human vaccination via the *i.m.*, *s.c.*, or *i.d.* routes results in a neutralizing antibody and T-cell response, though interestingly the antibody response was achieved at a much lower dose via the *i.d.* route [190]. ACAM3000 has also been shown to provide clinical and virological protection against Dryvax challenge [191].

IMVAMUNE (MVA-Bavarian Nordic) is another attenuated, replication-deficient vaccine in development. Several clinical studies have evaluated this vaccine for safety and immunogenicity [150, 186–188], resulting in the US FDA fast-track regulatory status of the vaccine. The absence of myopericarditis cases in more than 1900 vaccinations suggests that this vaccine may have an improved cardiac safety profile compared to ACAM2000® and Dryvax; however, more rigorous evaluation of this claim is needed. With regard to efficacy, vaccination with IMVAMUNE resulted in lower humoral and cell-mediated levels of immunity. Future studies are needed to determine if these are sufficient to provide durable protection [6, 189].

Therion Biologics also showed that an intramuscular MVA-based vaccine is safe and immunogenic and improves the safety and immunogenicity of subsequent Dryvax immunization [192]. Another attenuated, replication-deficient vaccine in clinical development is NYVAC, derived from the Copenhagen parent strain [193]. However, in clinical evaluations this strain was shown to induce suboptimal VACV-specific antibody responses [194].

3.7.4 Fourth-Generation Vaccines

Fourth-generation smallpox vaccines consist of subunits (nucleic acids, proteins) of VACV (Table 3.5). No fourth-generation vaccines have entered clinical development; however, we will briefly review them here, as they may have great safety advantages. On the contrary, subunit vaccines may be less able to induce the necessary immunological correlates of protection as the antigenic abundance within a subunit vaccine is substantially less than that of whole virus.

The work of Hooper and colleagues has shown that DNA vaccination with VACV genes elicits robust immunity and protection against MPXV in rhesus macaques [204–206]. Notably, they demonstrated that a four-component DNA vaccine could provide at least as much protection as MVA in nonhuman primates [207]. They further demonstrated that a recombinant subunit vaccine of VARV plasmid DNA followed by protein boost elicited protection against MPXV challenge [203]. Other groups have also shown protection with various DNA subunit combinations in mice [202, 208]. Buchman and colleagues showed that a protein subunit vaccine consisting of the A33/B5/L1/A27 gene products protected cynomolgus macaques from an otherwise lethal MPXV insult [201]. Several other groups have showed efficacy of protein subunit vaccines in VARV mouse models (Table 3.5).

3.7.5 Oral and Inactivated Vaccines

Oral and inactivated vaccines are relatively unexplored areas in the field. Bielinska and colleagues demonstrated protection in mice with inactivated VACV [209]. The inactivated vaccine Ospavir is derived from the parent Lister strain and gamma irradiated prior to administration [144]. In 1977, the vaccine was evaluated in a field trial using a prime-boost approach, with a prime intramuscular Ospavir dose followed by a first-generation Lister vaccine 1–7 days later [144]. The protective efficacy of this approach is unknown based on published data; however, Ospavir is licensed in Russia [144]. Other studies of inactivated vaccines from the twentieth century showed that although inactivated vaccines are capable of inducing humoral immunity, the antibody response many not provide sufficient protection [210–212].

Russia also developed a live oral vaccine near the end of the eradication campaign called TEOVac. The vaccine consisted of the B-51 strain of VACV and was shown to protect against smallpox disease after contact with smallpox patients. More recently, the oral vaccine was evaluated in a clinical trial and shown to induce a broadly neutralizing antibody response in all naïve individuals, with less success in vaccine-experienced individuals [144]. TEOVac remains licensed in Russia [213].

3.7.6 Global Stockpile of Smallpox Vaccines

At the conclusion of the SEP, a global stockpile was established for emergency use, under management of the WHO [6]. In 2004, WHO recommendations called for a stockpile of over 200 million doses of smallpox vaccine [214]. However, a simulated bioterrorism study demonstrated possible shortages in the event of an outbreak [39]. In 2013, the WHO Strategic Advisory Group of Experts on Immunization was asked to provide a recommendation for the stockpile size. In 2013, the stockpile consists of approximately 35 million vaccines; however, the advisory group predicted 600–700 million doses would be necessary for a response to an epidemic [142, 215].

First-generation vaccines make up a large portion of the global vaccine stockpile [141]. The NYCBOH and Lister strain are the source strains for most of the stockpiled first-generation vaccines. The Dryvax vaccine, produced by Wyeth, is no longer licensed in the United States due to a low supply and potential for contamination with adventitious agents from animal scarification [153]; however, it is still prominent in stockpiles throughout the world. The Aventis Pasteur vaccine, derived from the NYCBOH strain, is part of the US Strategic National Stockpile and together with several other vaccine products derived from the Lister strain composes a significant portion of the global stockpile [141, 142]. Second-generation vaccines that contribute to the global stockpile include ACAM2000® and the Lister-derived vaccines RIVM and Elstree-BN. ACAM2000® is US FDA-approved for those at high risk for smallpox disease and is part of the US Strategic National Stockpile [153]. ACAM2000® is also recommended for the WHO global stockpile [142]. The RIVM and Elstree-BN vaccines are stockpiled in countries throughout the world, and policy is in place for their emergency use [144]. The third-generation vaccine LC16m8 is stockpiled in Japan and also WHO-recommended for stockpiled efforts [142]. IMVAMUNE (MVA) is licensed in Europe and Canada [185] and part of the US national stockpile with investigational approval [153].

Clearly, smallpox vaccine development has become a large research activity that has output promising approved and investigational new-generation vaccines. As these vaccines reach regulatory approval, decisions regarding how many and which vaccines to stockpile must be made. Indeed, these have been a subject of recent discussion within the WHO [140].

3.8 Therapeutics

3.8.1 Overview

The risk for a smallpox bioterrorism event in tandem with endemic MPXV in Africa and outbreaks in the United States underscores the need for antiviral drugs toward smallpox and other orthopoxviruses. Notably, therapeutic strategies will likely involve combination therapy, especially because of concerns regarding drug resistance development. For instance, it was reported that VACV from a progressive

vaccinia case required the drugs ST-246, CMX001, and vaccinia immunoglobulin (VIG) for successful resolution because of resistance issues [216]. Thus, there is strong incentive to further progress virus- and host-directed therapeutics in the development pipeline.

3.8.2 Passive Immunization

Passive immunization in the context of smallpox has mainly been employed to treat complications of vaccinations [217, 218]. VIG is available from the CDC to treat complications of vaccination. The only documented uses of VIG in the prophylaxis or treatment of smallpox are from 1961, where VIG was used to prevent transmission of smallpox to contacts during an outbreak [219]. Otherwise, the efficacy of antibodies for treatment of orthopoxvirus infection in humans is unexplored. Several investigators have developed monoclonal antibodies with efficacy in animal models, but none of these have entered clinical evaluation [220, 221].

3.8.3 DNA Synthesis Inhibitors

Cidofovir is a dCMP analog that is indicated for the treatment of cytomegalovirus (CMV)-associated retinitis in AIDS patients; however, emergency protocols exist for its use to treat orthopoxvirus infections under an investigational new drug status [222]. Cidofovir targets the CMV DNA polymerase, a target shared by orthopoxviruses [223]. Multiple studies have demonstrated the anti-orthopoxvirus and protective activity of cidofovir, in both MPXV and VARV nonhuman primate models when administered within 48 h of exposure [224–226]. Due to nephrotoxicity associated with cidofovir, an oral formulation of the drug CMX001 (brincidofovir) was developed. This formulation allowed for uptake through lysophosphatidylcholine uptake pathways, resulting in less nephrotoxicity and improved bioavailability [227, 228]. CMX001 is currently in phase III clinical trials and has received FDA fast-track designation for indications of cytomegalovirus, adenovirus, and smallpox treatment. Notably, CMX001 recently received Orphan Drug Designation from the US FDA for treatment of smallpox. A variety of other DNA synthesis inhibitors, in the form of nucleoside analogs, are in preclinical development and are reviewed in [229, 230].

3.8.4 Viral Maturation Inhibitors

Quenelle et al. showed that thiosemicarbazones, used formerly as antituberculosis agents, inhibited VACV infections via inhibition of viral transcription and maturation [231]. Another anti-TB agent, rifampin, has been shown to inhibit the product of the D13 gene and block viral maturation in vitro [232]. However, high-dose

requirements and toxicities for treatment of orthopoxvirus infections with these drugs likely preclude their widespread clinical use [230]. Mitoxantrone, an anticancer agent with DNA replication and transcriptional inhibitory activities, has also been shown to block maturation of CPXV, MPXV, and VACV [233, 234]. Lastly, terameprocol, a phenol antioxidant, has also been shown to inhibit cell-to-cell spread of VACV in a variety of cell culture models and thus likely inhibits viral maturation as well [235].

3.8.5 Viral Egress Inhibitors

A high-throughput screen of over 300,000 compounds identified ST-246 (tecovirimat), which inhibits viral egress. The compound has activity against multiple members of the *orthopoxvirus* genus and is potent ($IC_{50} < 0.010$ μM) and selective ($CC_{50} > 40$ mM) [236]. Efficacy of this compound in multiple preclinical models, including one in nonhuman primates, has prompted safety evaluation of the compound in humans [237, 238]. The drug was recently FDA-approved for the treatment of smallpox and added to the US Strategic National Stockpile. The developmental progress of the drug is detailed in [239].

3.8.6 Host-Directed Drugs and Other Drugs

Several host targets are involved in orthopoxvirus replication, and efforts to target these have been productive in recent years. Host drug targets are attractive because drug resistance is less likely; however, more toxicity is expected. This must be tempered with the notion that use of host-directed therapies would be short term in nature for the treatment of smallpox infection.

The FDA-approved Gleevec (imatinib mesylate) and related drug dasatinib have been shown to impair the hijacking of actin motility by poxviruses and reduce infectivity in a mouse model [240, 241]. Additionally, EGF-like growth factors contained within poxviruses enable efficient viral replication. Targeting of these factors with CI-1033, a 4-anilinoquinazoline, reduced viral spread and showed efficacy in a VACV mouse model [242]. Other groups have shown antibody-mediated blocking of the EGFR can inhibit poxvirus spread in vitro [243].

In addition, advances in technologies for assessing host responses [49, 244] have also enabled the identification of several host kinase inhibitors inhibiting MPXV replication in vitro [245]. High-throughput host-directed small-molecule screens have also identified potent host-directed antiviral compounds active against orthopoxviruses and other viral infections. For example, FGI-104, identified in a high-throughput screen, has been shown to inhibit Ebola virus and CPXV infection, among others [246]. The mechanism underlying broad-spectrum host-directed antivirals remains to be fully elucidated. Interferon beta has also been shown to inhibit MPXV infection and spread [247]. Further safety and efficacy studies of host-directed drugs toward orthopoxviruses are warranted. Finally, in addition to host-

directed drugs, RNA interference has been demonstrated to inhibit MPXV replication in vitro [248].

3.8.7 Prophylaxis and Therapeutics: Future Directions

The prophylactic and therapeutic toolkit for orthopoxviruses has greatly expanded since the end of the SEP. Vaccines with improved safety profiles are available and beginning to be licensed, and promising therapeutics are on a regulatory fast-track pathway or were recently US FDA approved (ST-246, tecovirimat). Moreover, modern vaccine and drug development strategies are being used to fuel the orthopoxvirus drug and vaccine pipeline.

While motivating, these scientific endeavors come at a large public cost, as there is limited incentive for private drug development programs to develop drugs and vaccines for a disease that it is considered eradicated. Indeed, the large public cost of these research endeavors has been criticized in the scientific community and in the larger public [249]. At the same time, however, in an outbreak setting, a large vaccination campaign would likely result in many first-generation vaccine-associated deaths, especially in patients with relative contraindications, if large amounts of newer-generation vaccines are not stockpiled [144, 146]. Investment in orthopoxvirus drug development would further mitigate the risk of complications in patients with contraindications [146]. Therefore, future work and decisions must balance considerations of outbreak risk and costs of stockpiling.

3.9 Summary/Conclusions

There has been considerable debate as to the future of the remaining viable stocks of VARV following its global eradication. On one hand, there is an opportunity to destroy the last remnants of a virus that has claimed more human lives than all other infectious diseases combined. On the other hand, and as this review attests to, there is much that remains unknown regarding the molecular pathogenesis of VARV. Limitations to scientific research utilizing infectious virus have undoubtedly impeded our understanding of VARV transmission, life cycle, host restriction, and viral species-dependent pathogenesis (major vs. minor). Historical documents provide some context regarding clinical disease in humans; however, the molecular processes that underlie exacerbation or resolution of smallpox remain unknown. In addition, data from infection models in animals is questionable with the majority of infection models occurring prior to eradication and in the absence of advancements in clinical and research methodologies and infrastructure.

The recent accidental discovery of viable VARV stocks at the National Institutes of Health in the United States reignites the debate regarding the true accountability for all viable VARV stocks across the globe. Further, the recent reconstitution of the extinct horsepox virus using readily available technologies argues that a similar procedure could be employed to generate viable VARV for nefarious purposes

[27]. In addition, global climate changes have also been cited as a potential route for reintroduction of smallpox in humans due to thawing of permafrost where corpses from fatal smallpox were buried over a century ago [250]. Although the plausibility for any of these events can and should be debated, it does present a quandary regarding the continued preservation or destruction of remaining VARV stocks. To help guide this discussion and inform the research community, we have provided a thorough overview of current knowledge regarding VARV virology, clinical disease, pathogenesis, diagnostics, prophylaxis, and clinical interventions.

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Viral Therapeutics

4

Martin Richter

4.1 Introduction

Regardless of the described difficulties associated with antiviral strategies and drug development, some general antiviral strategies are: (1) inhibition of virion attachment to host cell membranes or membrane receptors, (2) inhibition of cell entry, (3) inhibition of viral nucleic acid synthesis and replication, (4) inhibition of viral protein synthesis, (5) inhibition of virion release [1, 2].

Due to the scope of this book, in the following subchapters, we will concentrate specifically on antiviral treatment options against viruses that are categorized to have a high potential of misuse and are therefore recognized as especially dangerous if used in biological attacks. The paradox of this statement, however, is that one important criterion for classifying a virus in this category is the lack of effective treatment (i.e. antivirals) or prophylaxis (i.e. vaccination). Currently, effective treatment options are available predominantly against viruses causing large public health burden, such as human immunodeficiency virus 1 (HIV-1), influenza A virus (FLUAV) or hepatitis C virus (HCV) and not against occasionally occurring high-consequence viruses (Table 4.1). One reason for this lack of medical countermeasures against viruses that can be used for biological attacks is the need of high containment (biosafety level 3 and 4)-facilities for handling viruses. Such facilities are difficult and expensive to operate as they must be equipped with highest security and safety standards. Thus, only a limited number of such facilities exist. In addition, socio-economic disparities have contributed significantly to lack of treatment options in the past [3, 4]. Recent incidents in structurally and economically weak regions show how fast a rare, yet lethal disease can become a worldwide major

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Table 4.1 Exemplary[a] treatment options for selected high consequence viruses

Agent	Disease	Treatment options (group of antiviral)
Variola virus	Smallpox	Cidofovir (nucleoside analogue)[b] Brincidofovir (nucleoside analogue)[b] Tecovirimat, (nucleoside analogue)[b]
Ebolavirus spp.[c]	Ebola virus disease	Hyper immune sera Convalescent human plasma[d] Recombinant human monoclonal antibodies (e.g. ZMapp) TKM-Ebola and small interfering RNA (siRNA) Phosphorodiamidate morpholino oligomers (PMO) Aphidicolin (DNA polymerase inhibitor) Brincidofovir (acyclic nucleoside phosphate—nucleoside analogue) Favipiravir (pyrazinecarboxamide—nucleoside analogue) Remdesivir and Galidesivir (adenosine analogues—nucleoside analogues) Cell entry and fusion inhibitors may also be considered as post exposure prophylaxis option when offered and authorized for compassionate use
VHF or severe disease causing bunyaviruses (Crim-Congo hemorrhagic fever virus, Hantaan virus, Andes virus, sin nombre virus)	Crimean-Congo hemorrhagic fever	Ribavirin (nucleoside analogue)[e] Passive antibody transfer Interferon therapy[f]
VHF causing arenaviruses (e.g. Lassa virus, Junín virus, Machupo virus and Chapare virus, Sabiá virus, Guanarito virus)	Lassa fever, Argentinian hemorrhagic fever, Bolivian hemorrhagic fever, Brasilian hemorrhagic fever, Venezuelan hemorrhagic fever, resp.	Ribavirin (nucleoside analogue)[e] Convalescent human plasma[d]

(continued)

Table 4.1 (continued)

Agent	Disease	Treatment options (group of antiviral)
Nipah virus	Acute encephalitis	Ribavirin (nucleoside analogue)[e]

[a]Depicted treatment options are exemplary and focus on agents that have been categorized as a biological threat if used in an attack. The list is not necessarily exhaustive. Overall substance-groups in parentheses and representatives are given. Note that specific representatives may vary internationally by derivative and brand. For dosage, dosage interval, combination, length of treatment and route of administration please refer to relevant national or international guidelines (e.g. WHO). Note that some depicted substances may have only restricted approval or no approval by the respective responsible national agency. Note that intensive care/supportive therapy in addition to medical treatment improves severe disease outcome and should always be considered. In cases of severe pulmonary involvement aggressive ventilation and extra-corporal membrane oxygenation (ECMO) therapy has also been used to improve outcome

[b]Effective against smallpox in laboratory settings and animal experiments; tolerated in healthy human beings; efficacy against smallpox in people unknown

[c]Treatment options depicted here have in part been used with some success in infected humans during previous ebolavirus disease outbreaks, mostly during the outbreak in Western Africa (2013–2016), causative agent EBOV in compassionate use attempts. In addition, all substances showed promising effects in animal studies in part leading to provisional, fast track, or orphan drug status designation approval by national pharmaceutical agencies in some countries. Full scale clinical trials are underway or have recently been completed with full conclusions often still pending

[d]Treatment efficacy dependent on titer levels of the specific neutralizing antibody

[e]Not approved for i.v. use in some countries (e.g. USA)

[f]Experimental, termination of treatment due to severe side effects in some patients reported

public health concern if medical countermeasures including treatment are practically non-existent. Especially the Ebola virus disease crisis in Western Africa in 2013–2016 was an intriguing example of this predicament. It took the local and international community more than 3 years to control and end this outbreak.

Other important factors for increased severe disease occurrence are habitat fragmentation, habitat alteration, human expansion, urbanisation and globalization [5, 6]. Especially globalization can be a contributor to cross boarder and cross continental disease spreading [5, 7]. Hence, preparation and establishment of countermeasures and an overall functioning health care infrastructure is adamant to contain agent transmission and ultimately disease to improve chances of survival.

However, the so called ebola crisis in Western Africa subsequently lead to increased efforts in the scientific and pharmaceutic communities to pursue existing treatment concepts into pharmaceutical formulations and novel promising approaches to develop effective treatments against Ebola virus Disease (EVD). In addition to the rather sparse existing treatment options against other high consequence viral diseases that could be used in a biological attack particular focus will be laid on some of these EVD treatment concepts in this chapter.

When discussing possible pharmaceutical countermeasures against high consequence diseases caused by viral agents with potential for use in a biological attack it is important to remember that there are today only few substances that can be considered. If they are available for a specific treatment such treatment is mainly,

with some exceptions, based on a single substance approach rather than a regimen as we have seen for instance in successful treatment against infections with dangerous bacteria or HIV. Regarding the positive experiences of a multi-substance approach with respect to multi targeting and anti-drug-resistance development it is advisable to also consider possible regimens for treatment against these high consequence viral infections as more substances become available

Regardless of specific treatment, it is important to note that recent studies and experiences in regions affected by the Ebola virus disease crisis show that critical supportive care and if available intensive care alone improve Ebola virus disease outcome and survival [8, 9] An important study on supportive care management of Ebola virus disease started in 2016 with a large panel comprised of physicians, practitioners and health care workers working in critical care, emergency medicine, infectious disease and general medicine, respectively, utilizing the concept of grading quality of evidence following GRADE (The Grading of Recommendations Assessment, Development and Evaluation working group)—guidelines from earlier reports, interviews and publications [10]. Further, public health experts and health research methodologists were also included in the panel and one psychologist, lawyer and bioethicist. The study was observed by WHO representatives. Study outcome was a thorough evaluation of specific evidence-based guidelines for supportive care suitable for EVD patients [10]. One of the first steps in treatment of high consequence viral diseases is according to this study rapid initiation of critical care measures such as fluid management and, where available, simultaneously or subsequently specific antiviral treatment.

As vaccination is not considered a classic treatment, existing vaccines against relevant viruses are discussed in the agent specific chapters of this book.

4.2 Inhibitors of Nucleic Acid Synthesis

The group of nucleic acid synthesis inhibitors is the largest and most important group of drugs for treatment of viruses and thus also for those that could potentially be used in biological attacks. Within this group we distinguish substances by their chemical features and by how they interfere with nucleic acid replication processes: (1) nucleoside analogues, (2) non-nucleoside inhibitors, and (3) pyrophosphate analogues [1, 2].

All representatives of the nucleoside analogues function as anti-metabolites and some of those in addition also act as nucleic acid chain terminations. Ultimately, these modes of actions lead to competitive inhibition of viral nucleic acid polymerases and subsequent complete inactivation of the enzyme and inhibition of 3′–5′ chain elongation, respectively. Structurally, nucleoside analogues are altered in composition of the pyrimidine or purine base or sugar component, respectively. Nucleoside kinases activate these anti-metabolites by phosphorylation to their respective tri-phosphates that are recognized by the polymerase and incorporated into the synthesized nascent strand. After incorporation, the polymerase is unable to detach and the enzyme is thus inactivated. Chain terminators received their name because they additionally inhibit strand elongation by lack of a hydroxyl group at the

3′ position of the sugar in their molecular structure, which is essential for nucleotide attachment to the nascent nucleic acid chain.

Novel representatives of this group also interfere with virion release, cause viral mutagenesis that specifically leads to reduced viral infectivity, and interfere with overwhelming host immune responses.

Non-nucleoside inhibitors allosterically inhibit nucleic acid polymerases near their substrate binding side. This inhibition prohibits substrates to access the catalytic active center of the enzyme and nucleic acid synthesis is prevented.

Pyrophosphate analogues inhibit nucleic acid polymerases by binding to the pyrophosphate acceptor side, which normally is responsible for pyrophosphate cleavage. This cleavage in principle enables nucleotide binding to the synthesized strand, which cannot be performed if the binding side is blocked.

Side effects of these substances can be very severe including impairment of respiratory function, drop in blood pressure, cardiac arrest, severe skin irritations such as Stevens-Johnson and Lyell syndromes, hepatotoxicity, and anemia. Side effects vary dependent on compound and application route and hence close patient monitoring is necessary. Many virostatics of this group are also substrates of cytochromes, like CYP3A4. Cytochromes are monooxygenases and play key roles in foreign substance metabolization. Especially if other medication is co-administered that is also a CYP-substrate, awareness of this side effect is necessary as one drug may competitively inhibit the degradation of another and therefore lead to potentially toxic elevated drug concentrations.

A special representative of the group of nucleoside analogues is ribavirin. This compound is specifically addressed here because it has been successfully used in treatment of mammarenavirus infections, which can result in severe hemorrhagic fevers such as Lassa fever [11] and in treatment of some orthohantavirus infections. Furthermore, the potential of ribavirin use in treatment of Crimean-Congo hemorrhagic fever (CCHF) is hotly debated [12]. Lassa virus, orthohantaviruses, and Crimean-Congo hemorrhagic fever virus are considered potential bioweapons agents and potential sources for clandestine use by non-state actors. Therefore, and because ribavirin seems to have an effect on at least three taxonomically distinct virus groups, ribavirin and its possible modes of action need to be further evaluated. The exact mechanism of action for ribavirin however remains elusive. Several suggestions have been made, among them inhibition of guanosine monophosphate and viral protein synthesis by interfering significantly in RNA capping events, which in principle is the attachment of modified guanosine to mRNA after transcription. Capping is adamant for starting the translation process in protein synthesis. It further suppresses humoral and cellular immune responses. In addition, more recent studies have also shown that ribavirin, when converted to a triphosphate, is preferably recognized by viral RNA-dependent RNA polymerases over cellular polymerase, leading to lethal mutagenesis in RNA virus genomes [13]. Ribavirin was for a long time the only agent with proven effectiveness against double stranded (mostly DNA) and single stranded (mostly RNA) viruses simultaneously which supports the theory of multiple mechanisms of action. Ribavirin is considered in national plans for stockpiling of antiviral agents for post exposure prophylaxis and treatment.

4.3 Recently Approved Substances and Substances for Provisional Use Against Potentially High Consequence Viral Infections

Tecovirimat (ST-246) is a 4-trifluoromethyl phenol derivative used in treatment of orthopoxvirus infections. It inhibits extracellular formation of viral particles by targeting specifically the F13L gene of orthopoxviruses. F13L encodes for p37, a highly conserved and peripheral membrane protein. p37 is required as integral part of a viral wrapping complex needed for envelopment and secretion of extracellular virus particles [14, 15] which cannot form if this protein is not present or non-functional. Tecovirimat thus does not function as a typical nucleoside analogue by inhibiting nucleic acid polymerases or acting as a chain terminator but functions as an inhibitor of virion release. Tecovirimat is active against a broad spectrum of orthopoxviruses including variola virus (causative agent of smallpox) [16, 17] and is part of national stockpile programs as a resource for post exposure prophylaxis and treatment. Tecovirimat was the first and remains so far the only antiviral ever to be approved under the FDA animal rule which was established in 2002. This regulation concerns the approval of new drugs when human efficacy studies are not ethical and if field trials are not feasible (e.g. after deliberately initiated exposure). Hence, drug approval is solely based on animal model studies.

Brincidofovir (BCV, CMX001) is a nucleoside analogue and a lipid conjugate of cidofovir. Cidofovir received US Food and Drug Administration (FDA) approval in 1996 for treatment of cytomegalovirus (CMV) retinitis in patients with AIDS. Similarly, BCV was initially developed as an antiviral against double stranded viruses [18] and clinical trials are ongoing to study efficacy against infections with CMV, adenoviruses, or variola virus. BCV also is active *in vitro* against Ebola viruses and has received investigational drug status by the FDA for treatment and clinical trials are underway.

Originally developed as an agent against influenza A virus [19], favipiravir (T-705) is active against Ebola virus (EBOV) in *in vitro* and *in vivo* experiments [20, 21]. Favipiravir is a pyrazinecarboxamide derivative and was, based on its nucleoside analogue structure, initially suggested to be a viral RNA polymerase inhibitor [19, 22]. Another proposed mechanism of action is that favipiravir induces viral mutagenesis that leads to limited viral replication and reduced infectivity [23]. Supported by these data, favipiravir has been considered in post-exposure treatment and after onset of symptoms for compassionate use against EBOV infections during the Ebola virus disease epidemic in Western Africa (2013–2016) and subsequently became subject to broad proof of concept studies and clinical trials [24]. As a result this study suggests that favipiravir monotherapy merits further study in patients with medium to high viremia, but not in those with very high viremia.

Other promising representatives are compounds galidesivir (BCX4430) and remdesivir (GS-5734). Both are nucleoside analogues that similar to other representatives of this group inhibit viral RNA polymerase function and cause chain termination. In animal experiments, they protected against EBOV infection even when administered intramuscularly after virus exposure [25–27]. From a drug

safety perspective it is noteworthy that galidesivir and remdesivir did not incorporate into human RNA or DNA underlining the drug's potential for approval, if clinical trials confirm animal experiments. Momentarily, use of galidesivir and remdesivir may be an option for compassionate use for potentially exposed individuals [26, 28]. Interestingly, remdesivir has recently also been found to show reasonable antiviral activity against more distantly related viruses such as Junín virus and Lassa virus [26], both mammarenaviruses causing high consequence disease and having the potential for misuse.

4.4 Inhibitors of Virus Attachment to Host Cell Membrane and Membrane Receptors, Inhibitors of Cell Entry

One of the first line defenses against infection of host cells is prevention of virion attachment to cell membranes and inhibition of cell entry. This concept has been successfully used in post-exposure prophylaxis against HIV-1 infections and as a supportive treatment option during anti-retroviral medication regimens. The option of inhibition of host cell membrane attachment and cell entry is therefore also an useful concept in treatment of other high consequence virus infections such as those caused by filoviruses (ebolaviruses, marburgviruses) and some contagious or highly virulent arenaviruses (e.g., Lassa virus, Junín virus), flaviviruses such as dengue viruses, and bunyaviruses such as Crimean-Congo hemorrhagic fever virus or several representatives from the family *Hantaviridae* (e.g., Hantaan virus, Andes virus, sin nombre virus).

FGI-103, FGI-104, and FGI-106 are all small molecule inhibitors of viral cell entry. Structurally they share heterocyclic aromatic structures (i.e., indole, benzofuran, benzimidazole, or benzothiophene backbones) connected via an aliphatic linker or directly to a phenyl substituent and possessing two positive-ionizable amidine or imidazolino moieties) [29]. FGI-103 and FGI-106 are structurally related to amiodaquine, an antimalaria compound, which together with chloroquine is active against ebolaviruses *in vitro* [30]. Both compounds are active against a broad spectrum of viruses even in animal models, but their mode of action remains unknown in detail [31, 32]. Some reports suggest an interaction of these compounds with the host vacuolar protein sorting (vps) machinery that is used by ebolaviruses for efficient budding [33]. EBOV replicates in macrophage/monocyte cells early in infection. An alternative or additional mode of action for FGI-103 and FGI-106 was suggested to be alteration of these immune cells, leading to inefficient replication of virus and thus providing more time for the host to concert an antigen-specific immunogenic response [29].

Further, a novel class of so-called fusion inhibitors has been derived from the lead compound LJ-001 (e.g., dUY11). Compounds of this class act as photosensitizers of viral membranes by generation of singlet oxygen ($^1O_2$) rendering them unable to fuse with host cell membranes and thereby preventing infection of host cells [32, 34]. These thiazolidine-based lipophilic broad-spectrum antiviral compounds

are effective against enveloped viruses only, but since most high-consequence viruses are enveloped, these compounds may be a promising treatment option.

A suggestion for further reading specifically with regards to advances in small molecule inhibitor research against infections with ebolaviruses is the report from Picazo and Giordanetto [35].

4.5 RNA-Silencing and Antisense Oligomer Therapy

Another promising approach is to inhibit viral messenger RNA by means of RNA silencing, an approach also known as RNA interference (RNAi). RNAi is a pathway present physiologically in most eukaryotic cells that uses small double stranded RNA with the purpose of gene de-regulation, gene knockdown or gene silencing. Two types of molecules are central to RNA interference, small interfering RNA (siRNA) and micro RNA (miRNA). In more general terms the RNAi system can be looked at as an "off-switch" for specific genes. The RNAi pathway is activated e.g. upon virus infection to interfere with virus replication as part of an innate response of the host organism.

siRNAs are ~21–22 bp long dsRNA molecules that display a 2 nt 3′ extension or overhang that allows them to be recognized by the enzymatic machinery of RNAi, which will then induce a so called RNA induced silencing complex (RISC) Activation of RISC eventually leads to gene knock down by directly targeting complementary mRNA structure and initiating degradation.

Another important molecule involved in RNAi is microRNA (miRNA). miRNA is an endogenous duplex that post-transcriptionally regulates gene expression by building a complex with RISC and subsequently binds to the 3′ untranslated regions (UTRs) of target sequences [36]. In general, the main mechanism of action of miRNA can be described as translational repression. In more detail, miRNAs are processed in the nucleus resulting in pre-miRNAs. These precursors are then exported to the cytoplasm, and processed further into active and functional miRNAs. miRNAs primarily inhibit translation via incomplete Watson-Crick base pairing to the 3′ untranslated regions of targeted mRNAs [36]. miRNA and siRNA pathways are interchangeable.

RNAi is a potent selective process with high fidelity. It has become an important methodology for targeted silencing of gene expression in mammalian cells.

In vertebrates and especially in mammals some viruses actively suppress RNA silencing features by encoding for so called RNA silencing suppressors (RSS). As a result these viruses can replicate at higher titer rates proving antiviral features of intact RNAi systems. Examples for viruses that encode for RSS are HIV-1 and EBOV which encode RSSs with equivalent activity [37, 38]. Utilizing described methodology and mode of action, RNAi molecules that target viral mRNA specifically and therefore inhibit viral protein synthesis and viral replication have been developed and successfully tested in animal studies. However, some candidates that looked promising in nonhuman primate studies, such as nanoparticle-based siRNAs (TKM-Ebola), did show little beneficial effect in human clinical trials and their

development was discontinued. The approach however is promising and should be clarified and evaluated further in complex organisms to elicit on its potential as a therapy option in humans.

4.6 Phosphorodiamidate Morpholino Oligomers (PMOs)

Especially in the field of drug discovery for substances against pathogenic filoviruses a small molecule therapeutics approach has also been successful in animal experiments and human trials. Phosphorodiamidate morpholino oligomers (PMOs) are antisense oligomers that target specific regions on viral mRNA that translate into filoviral proteins VP24, VP35, and RNA polymerase L, respectively, inhibiting virion production [39–41].

4.7 Targeting Hemorrhagic Disease Components

Aphidicolin belongs to the group of tetracyclic diterpenes and was isolated from fungus *Cephalosporum aphidicola*. Aphidicolin is a potent cell cycle inhibitor that interrupts DNA replication. The inhibitory properties enfold by targeting specifically B-family DNA polymerases, and therefore inhibit cell cycle transitions at the G1/S-phase step [42]. This feature leads to a dose-dependent decrease of EBOV infected cells. It received orphan drug status for the treatment of ebolavirus infections [43] by the FDA in 2016.

4.8 Immuno-Therapy

The approach of passive immune therapy with isolated immune globulin or treatment with convalescent plasma has been used successfully in the past and the former is well known as post-exposure treatment of animal bites to prevent rabies. Further, there are reports of successful use in treatment against high consequence infectious diseases such as CCHF and EVD and to some extend also against intoxinations with bio-toxins such as botulinum toxin. However, many studies lack proper efficacy assessments often due to limited availability of samples preventing sound conclusions [12]. In addition, stringent safety guidelines and measures need to be in place when acquiring and administering blood borne products. Especially in regions with high HIV-1, HCV or other chronic blood-borne pathogen prevalence, possibilities for acquiring sufficient amounts of uncontaminated immune globulin or convalescent plasma may be problematic and has to be subject to intensive donor screening and laboratory testing.

A combination of monoclonal antibodies against a specific agent often leads to better and faster treatment success than the use of one type of monoclonal antibody alone. An immunotherapeutic called ZMapp represents this approach with regards to treatment of EVD. ZMapp is a combination of two cocktails of monoclonal

antibodies that all bind to the core of EBOV glycoprotein [44]. ZMapp is associated with promising results in treatment of EVD patients, and the components of ZMapp can be produced through genetically modified tobacco plants [45].

4.9 Future Approaches

Pathogenesis of high consequence viral diseases often involve an overboarding inflammatory response of the host's immune system which can contribute significantly to severity of disease and is often among the hallmarks for disease and an important predictor of lethality [e.g., CCHF, or hantavirus pulmonary syndrome (HPS)]. When clearance of viral particles by the host immune response is unsuccessful, a subsequent reaction can be the excessive recruitment of pro-inflammatory cytokines, sometimes referred to as cytokine storm. This process is a fulminant and often life-threatening reaction of the host immune system as an overwhelming attempt to combat virus-infected cells. Therapeutic down-regulation of excessive concentrations of key pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) or interferons (IFNs) after infection may be a promising approach to prevent this life-threatening prodigious event. However, both innate and adaptive immune responses in appropriate strength are foremost protective measures of the host and inhibition of either for treatment purposes needs to be carefully evaluated.

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5 Challenges Associated with *Bacillus anthracis* as a Bio-threat Agent

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5.1 Introduction

Anthrax is a zoonotic disease caused by the gram-positive spore-forming bacterium *Bacillus anthracis* [1]. In nature, anthrax is usually a gazing animals' disease that can be transferred to carnivores feeding on the infected animals [2]. Infection of humans happens generally in developing countries, where sick farm animals are quickly slaughtered to salvage the meat [2, 3]. Usually in these cases, the owner then feeds the family, the neighbors and in some cases the family pets, causing gastrointestinal anthrax that, if left untreated, results in death [4, 5]. Under the same circumstances, direct skin-contact with contaminated blood commonly results in cutaneous anthrax. This is a relatively moderate form of the disease that is fatal only in about 30% of the cases when left untreated [2]. A third, rare way to "naturally" contract anthrax is spore inhalation [6]. Inhalational anthrax was so rare that it was considered an occupational disease, mainly in goat hair-processing mill workers who contracted the disease from aerosolized *B. anthracis* spores contained in infected animal products [6, 7]. Hard to diagnose and rare, inhalational anthrax is fatal in most of the cases [2]. This form of anthrax is the main threat to populations exposed to a malicious release of *B. anthracis* spores.

B. anthracis is closely related to *B. cereus*, a human pathogen that is generally associated with food poisoning, and *B. thuringiensis*, a specific pathogen of insects [8]. Like many closely related bacilli, *B.* anthracis is a soil bacterium, probably commonly present in the stable form of spores rather than as vegetative bacteria [8]. As the spores enter a susceptible host, they germinate and start an infection cycle that will result in the death of the host or the elimination of the bacteria (obligate

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pathogen) [2], as there are no reports of chronic anthrax. The death of the (non-human) host is usually accompanied by massive nasal and rectal hemorrhages that distribute massive amounts of bacilli to the environment [9]. Once exposed to oxygen and ambient temperatures, the bacilli start to sporulate, forming highly durable spores that persist in the environment, waiting to infect the next host [9].

Infection of a host occurs when a spore penetrates through abrasions in the skin or across the lung epithelium [2]. In the skin, the spore germinates at the point of entry, inducing a local immune response that can restrain the bacteria from progressing to a systemic infection. Systemic infection is initiated when spores are picked up by a phagocytic cell and transferred to a draining lymph node. The germinated bacilli overcome the immune cells by encapsulation and toxin secretion, escape the lymph node to spread systemically, causing a lethal systemic infection. In the case of gastrointestinal route, the host is infected by bacilli from contaminated meat or by environmental spores. The bacilli then cross the intestinal lining into the blood-stream, leading to systemic infection and death [2].

The virulence of *B. anthracis* relies on two major virulence factors; the tripartite toxins [10] (encoded by the virulence plasmid pXO1) and the antiphagocytic capsule [11] (encoded by the virulence plasmid pXO2). The toxins are composed of two enzymes and a pore-forming protein: the metalloprotease lethal factor (LF), the calmodulin-dependent adenylate cyclase edema factor (EF) and the pore-forming protective antigen (PA). PA, which is the most abundant of the three proteins, binds to specific receptors located on the surface of all mammalian cells, is processed by a host protease (furin) and oligomerizes to form a heptamer, presenting three LF/EF binding sites. This heptamer usually binds LF and EF in a 2 to 1 ratio. The complex is then internalized into a phagosome that gets acidified following lysosomal fusion. This acidification causes a conformational change in the structure of the PA heptamer, injecting the bound LF and EF into the host cell's cytoplasm. LF cleaves most of the MAP kinases, whereas EF, which is one of the most potent adenylate cyclases characterized, causes a dramatic increase in cAMP concentration. The result is a significant disruption of cell signaling and regulation. The two most dramatic outcomes of these toxins' activity are inactivation of the host immune response and modification of endothelial permeability [10, 12].

Several incidents of accidental human anthrax were documented since the early 1900's. In a case only recently attributed to anthrax, horse-hair shaving brushes made of infected animals' hair were supplied to US and British troops causing infections [13]. The best documented case is the anthrax outbreak in goat hair-processing mills in Pennsylvania and New Hampshire [14]. These cases were examined carefully and the data from these events still serve as the source of the maximal daily exposure dose of spores that is safe and will not cause anthrax (~600/day) [14]. Following this event, specific protocols were imposed that include wearing face masks and vaccination of at risk workers [15]. The studies that followed these events set the foundation for the determination of the physical properties of the infective particle, demonstrating that the most infective particle size was around 3 microns in diameter [14, 16, 17]. This size indicates that the site of infection is the lower respiratory tract. The dose which will result in the death of 50% of the infected population (defined as Lethal Dose 50, or LD_{50}) is still unknown. The

fact that the reported LD_{50} of inhaled spores in animal models is in the rage of 10^4 to 10^5 CFU, regardless of animal size (guinea pigs to non-human primates) [17] might imply that humans fall within the same range. The most common estimated LD_{50} dose for humans is ~10^4 CFU [18]. The WHO estimates that a deliberate, military discharge of 20 kg of spores over a city of 2.1 million will result in the death of approximately 250,000 people and cause 20 billion dollars in damages [18]. In the case of a bio-terror attack, the discharged amount is assumed to be considerably lower and so will be the number of casualties. Nevertheless, the economic damage might be the same [19], or even higher since it is assumed that a bio-terror attack will go undetected for a longer time, resulting in delayed containment actions. This delay would result in a more significant spore carryover, especially in highly populated cities, leading to larger contaminated areas. A notion of the logistics that are associated with a single discharge of *B. anthracis* spores over a populated city can be gained from the accidental release of spores from a military facility in 1979 in the Soviet city of Sverdlovsk (currently Yekaterinburg) [20, 21]. A presumably misplaced HEPA filter in one of the ventilation systems resulted in the discharge of aerosol containing an unknown number of weapons-grade spores into the atmosphere. Luckily the wind direction was away from the city. Even though the authorities were aware of the accident and recognized that the patients that started to accumulate had anthrax, at least 66 people died despite antibiotic treatment. Farm animals died at a distance of more than 50 km from the city. A general vaccination program for people aged 18 to 55 who were considered at risk, was initiated (about 55,000 civilians) [20].

B. anthracis spores were used in at least two documented bio-terror events. The first was the discharge of spores by the Japanese Aum Shinrikyo doomsday cult [22]. They discharged B. anthracis spores from the top of the cult building in Tokyo, an event that was unnoticed since the strain that was used was a vaccine non-encapsulated strain (Sterne) [22]. This strain is nonpathogenic for humans but will kill mice, presumably the animal model that was used to test the *B. anthracis* strain by the cult.

In the second (and deadly) bio-terror attack in 2001, anthrax spore powder was mailed on two occasions in probably seven sealed envelopes to various newspapers and governmental offices in Florida, New York, and Washington, DC ("Amerithrax") [23]. The efficiency of this simple attack was enhanced by the automatic mail sorting machines, which generated a spore aerosol that contaminated the mail sorting buildings, cross-contaminating additional mail items [24], and expanding the impact of the attack. Among the first victims were people who opened the envelopes, followed by postal workers that were exposed to the aerosol and the contaminated mail [23]. Only four of the envelopes were recovered, and the identity of the person(s) behind the act remains controversial [25].

One of the immediate responses to spore exposure is antibiotic treatment of populations at risk. All forms of anthrax can be efficiently treated with antibiotics, if the treatment is initiated early enough, preferably before the onset of clinical signs [18, 26]. In the 2001 attack, the first anthrax diagnosis was of patient 8, who suffered from inhalational anthrax [23]. This patient was suffering from meningitis and was

treated for bacterial meningitis, when the cerebrospinal fluid (CSF) sample revealed gram-positive bacilli and alerted the care giver to the possibility of anthrax [27]. Prior to that case, there were six cutaneous cases (cases 1–3 and 5–7) that are considerably easier to diagnose [23]. Since anthrax is rare, none of the medical personnel involved had ever seen an anthrax patient and none knew exactly how to diagnose or treat the disease. In total, this event included 22 patients, 11 with inhalational anthrax and 11 with cutaneous anthrax. About 50% of the patients with inhalational anthrax were diagnosed too late for treatment to be effective and succumbed to the disease; some had been misdiagnosed early on and discharged from the hospital [27]. The enforcement of standard effective diagnosis and treatment protocols dramatically reduced the number of casualties [28]. Rapid methods for detection, identification of people who were exposed, and the mapping of contaminated areas enabled initiation of post-exposure prophylactic treatment and cleaning procedures that probably prevented the escalation of this event and the further infection of additional people [29]. These decontamination efforts were complicated by the nature of anthrax spores, i.e. their extreme resistance to extreme environmental conditions such as high temperature and drought and their ability to persist in the environment for years without losing their viability and infectivity. Therefore, identification and confinement of the contaminated area is essential to ensure proper and effective decontamination. Public areas such as the US senate or the New York NBC offices required expedited and expensive decontamination procedures that lasted months. Decontamination of the central post offices took years [29].

The search for the person(s) who conducted this attack also took years and involved the development of sensitive genetic tools and analysis of hundreds of strains from laboratories all over the United States. Eventually no one was convicted and following the suicide of a senior scientist at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), controversial evidence was published implicating him as the one who mailed the anthrax letters. The investigators singled out a USAMRIID batch as the source of spores [25].

Herein we will discuss the challenges associated with the use of *B. anthracis* spores as a bio-terror agent and ways to counteract them. We will go through the different challenges of patient diagnosis and treatment. We will discuss the challenge of monitoring the environment and decontamination. We will describe the available forensic tools and discuss the challenges of identifying spore production prior to the dissemination.

5.2 Diagnostics (Clinical)

A major challenge for the physician is the diagnosis of “patient 1” of inhalational anthrax. In the absence of the typical skin lesion (eschar) of the cutaneous form or the diarrhea of the gastrointestinal form, the first clinical signs of the inhalational disease are reminiscent of influenza [6, 27]. Inhalational anthrax is a two-phase disease [2, 18, 30]. Following exposure, during the incubation period, the patient

develops influenza-like clinical signs and symptoms that include fever, malaise, fatigue, myalgia, coughing, headaches, vomiting, chills and abdominal and chest pain. This stage, lasting from several hours to a few days (usually 2 to 5 days), is occasionally followed by a brief period of recovery. The acute phase which follows consists of high fiver, dyspnea, stridor, cyanosis, diaphoresis, severe respiratory distress, shock, and finally death. Data from animal models indicate signs such as hypocalcemia, extreme hypoglycemia, hyperkalemia, depression of respiratory centers, hypotension, anoxia, respiratory alkalosis, and terminal acidosis. Case reports from 10 of the 11-inhalational anthrax victims from 2001 [27] revealed lymphadenopathy and enlargement of the mediastinum that led to stridor. Of the 10 reported cases, all had abnormal chest X-ray images, seven had mediastinal expansion, seven had infiltrates, and eight had pleural effusions. Autopsy data from the Sverdlovsk incident showed a substantial amount of necrotizing hemorrhagic pneumonitis, hemorrhagic mediastinitis, pleural effusions (1000 ml in quantity on average), and hemorrhagic meningitis [31].

Since inhalational anthrax is rare, most clinical data derived from animal model experiments. Though experimental anthrax models were developed to include laboratory mice, guinea pigs, rabbits, and non-human primates (NHP) for different purposes [32, 33], the US FDA acknowledged rabbits and NHP as the relevant animal models for the disease [34]. *B. anthracis*-induced clinical signs in animal models are not exactly identical to human clinical signs. Unlike the two-phase disease in humans, in rabbits and NHP [32, 33, 35], following the asymptomatic incubation period, anthrax manifests in a single acute phase, hours prior to death. These symptoms include fatigue and severe respiratory distress leading to death. Autopsy of rabbits and NHP reveals massive damage to the internal organs and often hemorrhagic meningitis [36–38]. Similar to humans, the best marker for anthrax in animal is the presence of bacteremia—bacilli in the blood [35, 39]. Bacteremia is indicative of the systemic phase and the level of bacteremia is a parameter of the severity of the disease, at least in animal models. Experiments in rabbits and NHP indicate that the antibiotic treatment is highly effective when administered to animals with bacteremia of up to 10^4 CFU/ml. The efficacy of the treatment decreases with the increase in bacteremia to a point when the antibiotic is not effective any longer [39–42]. The cut-off bacteremia titer at which the treatment is not effective depends on the antibiotic. In rabbits it ranges from 10^5 CFU/ml to 6×10^6 CFU/ml [41]. The main drawback of using bacteremia as an indication of anthrax is that classical means of detection are cultures that require 12 to 16 h to display a positive result, during which time the progression of the disease continues and the therapeutic window closes. A way to overcome this problem was the identification of specific biomarkers in animal models that are easy to detect and that correlate with the bacteremia titers. The blood level of several bacterial derived proteins seemed to correlate well with the bacteremia titer, two of them are toxin components; PA and LF. PA can be detected using an antibody-based test and is present in ng/ml concentrations at initiation of bacteremia and μg/ml at the late stages of the disease [35]. LF on the other hand, is detected by an activity-based test [43]. Since LF is a metalloprotease with a specific cleavage site in the target MAP kinase, the test is

based on the specific cleavage products of a short peptide that are detected by a mass-spectrometry analysis. The sensitivity of this activity test is at the range of ng/ml and the level of activity that was detected in patients is at the range of 10 ng/ml to 1 μg/ml [43]. The typical δ-poly-D-glutamic acid capsule (although not exclusive to *B. anthracis*) is a potential marker for anthrax. This capsule polymer is released into the serum and could be detected by simple immunoassays such as lateral flow [44]. The fact that closely related *Bacillus* strains produce a similar polymer does not affect the specificity of this marker since these strains are non-pathogenic, making their existence in the bloodstream highly unlikely.

Diagnosis of inhalational anthrax according to the US CDC is based on the confirmation of mediastinal widening and pleural effusion by chest X-rays or CT scans [18, 30]. The only ways to confirm an anthrax diagnosis are to detect circulating toxins in blood and/or to directly identify *B. anthracis* in any of the following samples: blood, skin lesion swab, spinal fluid or respiratory secretions. These samples must be taken prior to the initiation of an antibiotic treatment. The 2010 US CDC case definition [45] defines three case classifications; suspected, probable, and confirmed. **Suspected** refers to illness suggestive of one of the known anthrax clinical forms, without any evidence of *B. anthracis* or epidemiologic evidence related to anthrax. A **probable** case is one that was not confirmed but is either epidemiologically linked to a documented *B. anthracis* environmental exposure or found positive for *B. anthracis* DNA, LF activity, specific cell wall antigens or PA-specific antibodies in usually sterile clinical samples. Al these tests must be performed by a certified laboratory performing approved tests (PCR, Quick ELISA Anthrax-PA kit, LF MS, and Redline Alert test). A **confirmed** case is defined as one presenting one of the following: (a) Association with culture and identification of *B. anthracis* from clinical samples by one of about 140 US Laboratory Response Network (LRN) laboratories. (b) Demonstration of *B. anthracis* by specific immune-histochemical stain of tissue samples. (c) Evidence for four-fold increase in anti-PA specific IgG using a CDC quantitative ELISA testing [46]. (d) Documented *B. anthracis* environmental exposure and evidence of *B. anthracis* DNA (by LRN validated PCR) of usually sterile samples (blood or CSF).

The definition of a case as "suspected" is the critical step in this process since wrong diagnosis at this stage can result in mistreatment of the patient and death. Other than indications for mediastinal widening and pleural effusion that are not anthrax-specific, the caregiver has no tools other than his own experience to suspect anthrax. In the case of the 2001 cases, other than mediastinal widening and pleural effusion, one of the main pointers towards anthrax was a positive blood culture with gram-positive bacilli [27]. Being a US Tier 1 Select Agent, the consequences of false-positive results of a diagnostic test, performed by an uncertified laboratory is at least undesirable. However, rapid diagnosis and initiation of the right treatment are crucial for the survival of an anthrax patient. Bed-side testing for *B. anthracis*-secreted antigens such as PA, LF, and/or capsule, could give a positive, specific indication to the presence of the bacilli in blood samples, assisting the caregiver in providing the best care for the patient.

Undoubtedly, the challenge in the diagnosis of patient 1 following *B. anthracis* release in a bio-terror event would hinge upon medical staff awareness which, without indications to the contrary, would intuitively correlate initial inhalational anthrax symptoms with common seasonal illnesses. This challenge requires constant briefing of first responders to elevate awareness to the symptoms of the different bio-threats, since patient 1 may possibly be the first indication of such an event.

5.3 Treatment

Post exposure treatment can be divided into two categories with respect to symptoms onset. "Post-exposure prophylaxis" (PEP) is a preventive treatment of individuals who are at high risk of exposure or were exposed, but have not yet develop symptoms. "Treatment" is usually referred to as treating symptomatic patients [18, 47].

The treatment's efficacy is in inverse correlation with the time between exposure and treatment initiation; the closer the initiation of treatment to the exposure, the more likely the treatment will be effective. In 2001, none of the people who received post-exposure prophylaxis consisting of oral administration of ciprofloxacin or doxycycline developed systemic anthrax [23]. Unfortunately, of the 11 patients who arrived at the hospital during the acute phase of the disease, five arrived too late for efficient treatment and died [23, 27]. Death was due to the extensive systemic damage in some cases or non-effective antibiotic treatment that did not prevent deterioration. Tragically, a few people sought out medical attention and were discharged from the hospital without treatment, misdiagnosed as suffering from influenza [27]. The surviving patients were discharged from the hospital after about a month and only one of them returned to his previous place of employment.

All of the *B. anthracis* strains that were isolated so far from nature are sensitive to most antibiotics according to in vitro tests [48]. The efficacy of different antibiotic treatments was tested in animal models from laboratory mice and guinea pigs to rabbits and non-human primates [18, 40–42, 49]. The efficacy of post-exposure prophylaxis of doxycycline and ciprofloxacin was demonstrated in all animal models. Although a short antibiotic treatment was very effective in preventing death, upon cessation of treatment about 50% of the animals relapsed and died [39, 40, 42]. This relapse is typical to inhalational anthrax and is due to a "spore depot" that remains in the lung during antibiotic treatment and germinates upon the cessation and decrease of circulating antibiotic concentrations. Therefore, US CDC recommendations for PEP are 60 days of ciprofloxacin or doxycycline with a strong recommendation for administering a PA-based vaccine (AVA, Biothrax) [30].

Testing treatment efficacy in relevant animal models, rabbit and NHP, is based on the only reliable indication for *B. anthracis* infection—blood bacteremia [35, 39]. Body temperature could also be used as a marker for systemic disease in animal models with two main limitations/reservations; firstly, temperature should be measured continuously since occasionally the body temperature drops at the final stages of the systemic disease [50, 51]. Secondly, temperature can indicate systemic

bacillus spread but cannot indicate bacteremia titers or the severity of the disease. In most animal experiments, single drug treatments were evaluated. The efficacy of fluoroquinolones was high in all animal models, with ciprofloxacin, moxifloxacin and levofloxacin being the main drugs tested from this family. These drugs showed high prophylactic and reasonable systemic treatment efficacy [39, 42, 52]. In humans, ciprofloxacin prophylaxis was administrated in 2001 to prevent the onset of anthrax in people who were confirmed or at risk of exposure to *B. anthracis* spores [53]. During the 1979 Sverdlovsk incident, tetracycline was the antibiotic of choice in treating exposed populations [20]. In both cases, the prophylactic treatment was very effective. Historically, anthrax was treated first with sulfa-drugs, and later on with streptomycin, penicillin G, and erythromycin [54]. The finding that *B. anthracis* encodes β-lactamases restricted the use of penicillin G (and other β-lactams) to cases infected with sensitive strains [47]. Hence, β-lactams are only as a second choice for prophylactic treatment and the US CDC recommendations for post-exposure prophylaxis are fluoroquinolones, mainly ciprofloxacin, or tetracyclines, mainly doxycycline [47]. The treatment of systemic patients is more complex. Since anthrax could and was diagnosed as atypical pneumonia, the drug of choice was usually a cephalosporin [27]. As subfamily of β-lactams, *B. anthracis* is generally resistant to cephalosporins due to presence of inducible resistance genes. Thus, in vivo induction of these resistance genes results in treatment failure and death if the drug is not changed [27]. In 2001, systemic anthrax was successfully treated using a combined treatment of ciprofloxacin and clindamycin. Accordingly, the US CDC recommendation for treatment was this combination [27, 55]. In many cases in 2001, this drug combination was expanded by additional drugs [55]. In some cases the treatment included up to nine drugs together. The ciprofloxacin/clindamycin combination, though very efficacious, has its flaws; a major one is emergence of *Clostridium difficile* infections. In addition, central nervous system (CNS) infections were demonstrated in animal models [36, 37, 51] and humans, usually post mortem [18, 31, 56]. In 2001, at least one of the patients (Case 8) had CNS infection as judged by biochemical tests and gram stains of CSF samples [27]. In data recovered from the 1979 Sverdlovsk incident, in 39 of 42 autopsies, records of cerebral hemorrhage (“a cardinal’s cap”) were scored [31]. The same phenomenon was documented in NHP [36], indicating that anthrax treatment must include a treatment for CNS infection i.e. meningitis. Recent CDC recommendations [47, 57] address this point and state that unless definitively excluded, one has to consider all anthrax patients as suffering from meningitis. However, unlike the previous recommendations that relied, at least partially on case reports of anthrax patients, the new recommendations include treatments used in other non-anthrax CNS infections [47]. These recommendations include the combination of two to three antibiotics with an anti-toxin treatment and dexamethasone in the case of meningitis. The antibiotic treatment relies on the combination of a fluoroquinolone (levofloxacin or ciprofloxacin) and a protein inhibitor (linezolid or clindamycin). In the case of meningitis, a third antimicrobial β-lactam (meropenem or imipenem) is to be added to the treatment [47, 57]. The use of antitoxin-antibodies was previously documented in an inhalational anthrax patient. In this case a polyclonal antibody

preparation purified from Biothrax (AVA) vaccinated volunteers served as antitoxin. Recently, at least three additional antitoxin drugs have been approved by the FDA for treating anthrax, all of them are anti PA neutralizing human monoclonal antibodies [58]. Though tested extensively in animal model, the contribution of adding the antitoxins to the combined antibiotic treatment has never been demonstrated [59]. Nevertheless, we demonstrated in rabbits that at the systemic stage of anthrax the toxins are redundant and therefore the administration of antitoxins should be reconsidered on the base of cost efficiency, especially when the failure of these treatments was correlated to CNS lesions [60].

The first-choice antibiotic treatment is the combination of levofloxacin and linezolid [47, 57]. The efficacy of levofloxacin in combination with additional antibiotics was demonstrated at 2001, though never shown to be superior to the less expensive ciprofloxacin. Linezolid on the other hand was never used in anthrax patients. Additionally, clindamycin, which is recommended as second choice, was part of the successful treatment of several anthrax patients [27]. The efficacy of linezolid treatment of anthrax was demonstrated in animal models but never in combination with fluoroquinolone, as was the combination with clindamycin [41]. The addition of a carbapenem (meropenem or imipenem) as a β-lactam to the treatment in the case of meningitis was similarly never tested. β-lactams are considered a good treatment for CNS infections due to the relatively high blood brain barrier (BBB) penetration. The choice between ampicillins versus carbapenems was never addressed and in the case of *B. anthracis* the use of the new generation of drugs may not be superior to the previous one.

B. anthracis is genetically stable. The fast rate of infection progression from infection to blood stream invasion causing a systemic homogenous infection leading to rapid killing of the host and then returning to the dormant spore stage (sporulation) limits the opportunity of gaining antibiotic resistance genes by horizontal transfer from other bacteria that usually reside in the gut. Therefore, the recommended antibiotic treatment can rely on the well-established list with documented efficacy in treating anthrax in humans or relevant animal models. Fortunately, unlike in the case of the naturally occurring multidrug resistant strain of *Yersinia pestis*, in this case the list is long enough to cover spontaneous or deliberate antibiotic resistance of a specific strain [41].

Though antibiotic treatment seems to play a major role, the 2001 case reports demonstrate that when patients did not receive proper supportive care, the efficacy of the antibiotic treatment was dramatically reduced [27]. Pleural effusions were present in all patients that were admitted to the hospital. In 7 of them, these pleural fluids were drained, up to a total volume of about 3 l per patient. Out of these 7, 6 that had been treated with *B. anthracis*-effective antibiotics recovered [27].

The absence of antibiotic-resistant *B. anthracis* strains in nature [48] is not an assurance that an antibiotic resistant strain will not be used in a bio-terror attack. Generating antibiotic resistant strains is relatively simple but requires microbiological skills and equipment. However this process can result in the attenuation of the new strain, a change that is undesirable for the terrorist. Therefore, efforts to develop combined procedures that will include antibiotic and

non-antibiotic treatments must be advanced to determine effective protocols for both antibiotic-sensitive and -resistant strains. Targets could include the inhibition of specific metal transporters, iron for example, or specific adhesins or sortases that are involved in the localization of proteins on the surface of the bacilli. These inhibitors mast be specific to the bacilli and should not interfere with similar processes of the mammalian cells.

5.4 Epidemiology and Environmental Diagnostics

In 2001, the first batch of spore-containing letters was mailed on September 18th, causing nine cases of cutaneous anthrax and only two cases of inhalational anthrax [23]. These two patients were admitted to a Florida hospital on the 1st and 2nd of October. The first anthrax-related death of one of these patients occurred on October 5th. All of the cutaneous cases were reported in New York and New Jersey. The second and deadlier batch of letters was presumably mailed on October 9th. This batch directly caused seven (presumably nine) inhalational anthrax cases and one (presumably two) cutaneous cases [23]. All the cases of inhalational anthrax were admitted to hospitals in the New York area. Symptoms were documented as early as October 14th and the first case arriving at a hospital was documented on October 19th [27]. Three of the cases sought out medical assistance and were discharged with the wrong diagnosis [27]. Only in one of these cases was a blood culture taken and upon receiving positive growth results was the patient started on antibiotic treatment at home. That treatment was only partially effective since the patient was readmitted to the hospital due to his deteriorating condition two days after treatment initiation [27]. The other two patients returned to the hospital too late and they succumbed to the infection on October 21st and 22nd [27]. A forth case was a New York City hospital worker who started showing symptoms on October 25th. Her symptoms progressed until she was admitted to the hospital on October 28th. This case was misdiagnosed and treated for atypical pneumonia. Diagnosis of anthrax was made by positive blood culture after her death on October 31st [27].

Since inhalational anthrax cases are extremely rare, any reported case must raise an alarm, initiating an immediate epidemiologic investigation. At the same time, the health care community and medical centers have to be notified that such a case has been documented. These facilities must be made aware of the specific and nonspecific symptoms of the disease and should report any suspected case to the authorities [61]. In retrospect, three of the five casualties during the 2001 Amerithrax episode might have been saved if such measures were taken. Epidemiological studies revealed that most of the inhalational cases worked in US postal facilities, strongly indicating those facilities as the sites of exposure [62]. This finding enabled the start of effective preventive treatment of other workers at risk of exposure prior to development of symptoms by any of them [29]. At the same time, identification of the exposure sited enabled initiation of containment and decontamination efforts.

Identification of an attack is highly dependent on the type of bio-terror event; silent or public. In the case of a silent release of spores, the first indication of the

event will be the appearance of patients at points of care seeking medical attention (as in 2001). Taking into consideration person-to-person differences in incubation time, cross-referencing the whereabouts of the different patients might give an indication of the site of attack. For example, in the 2001 event, the patients were postal workers who worked in two facilities and were hospitalized during the same time frame, indicating that the postal facilities were contaminated and served as the site of exposure [23, 62]. On the other hand, the patients from the New York City hospital and Connecticut were not connected to any of the other cases and were not related to any of the known anthrax letters [23], leaving their exposure location unknown [25]. Furthermore, the anthrax envelopes were clearly connected, as all of them contained powder and a threat letter [25]. In one case, the recommended treatment was clearly indicated in the letter in case the recipient did not know how to respond. In this case, isolation and identification of the *B. anthracis* spores was relatively straight forward (having an explicit threat to focus powder identification efforts. In addition, forensic efforts should allow to identify the sender. Although the letters that were sent on October 9th caused significant economic collateral damage, no one in the Senate offices contracted anthrax [29]. The arrival of powder-containing mail alerted the recipient and initiated antibiotic prophylaxis, preventing the development of any form of the disease [29, 63]. The public awareness that followed and the world-wide news coverage of the event was so substantial that to this day, receiving a powder-containing letter will probably raise a red flag anywhere. This awareness resulted in immediate containment and the initiation of mapping exposed personnel, allowing swift and effective treatment.

Environmental monitoring as part of the epidemiological investigation and during follow-up and validation of the decontamination procedure is based on the detection of *B. anthracis* spores. Since spores survive in the environment for years [64], the challenge is locating the contaminated areas and collecting a sufficient amount of spores to enable detection. In cases associated with visible powder residue, sampling will usually involve various swabs—cotton, polyester, nylon or different macro-foam materials [23, 29]. The most effective sampling method was the use of a vacuum cleaner that traps the spores on HEPA filters [29]. Spores are extracted from these materials with liquid solutions containing salts and/or detergents [24]. The classical microbiological detection methods will include plating on different agar media selective for *B. anthracis*, or rich blood-containing media [56, 65]. Alternatively, identification can rely on testing the sensitivity of the isolate to γ-phage, a bacteriophage specific for *B. anthracis* [56]. Since these tests rely on the viability and growth of the spores, the microbiological methods are slow, taking up to 48 h until a final positive result is obtained. Though a microbiological test is essential to determine that an intentional discharge of viable *B. anthracis* spores had occurred, the rapid tests will rely on polymerase chain reactions (PCR) [66] or specific anti-spore antibodies [67]. The challenge of rapid testing is specificity. *B. anthracis* is genomically almost identical to other bacilli in the *B. cereus*/*thuringiensis* group. The DNA-based tests usually rely on the detection of virulence plasmids in general (pXO1 and pXO2), specifically the genes for the toxin (*pag*, *lef*, and *cya*) and capsule (*capA–E*) [66, 68]. The combination of the two enables the

discrimination between the live attenuated Sterne like anthrax vaccine ($pXO1^+$, $pXO2^-$) and the virulent *B. anthracis* strains that contain the two plasmids. It is important to remember that the specificity of PCR tests is based on the primer sequences and their specificity ability to enhance *B. anthracis* genes while not recognizing other organisms' DNA. This specificity is determined by sequence analysis of genomes in the GenBank, overlooking other organisms that exist in the environment but may not be represented in the GenBank. In addition, the major advantage of the PCR test is also its vulnerability. Because the test is so sensitive, the risk of contamination or a false positive result from partially homologous DNA is high. Therefore, this test must be performed by a certified laboratory performing standardized tests with the appropriate negative and positive controls [18, 30].

Antibody based tests are common for a variety of applications, from testing for pregnancy to the identification of pathogens in different environmental, food, and clinical samples [69, 70]. The key for such a test is the specificity of reagents (antibodies). Since most of the spore proteins on *B. anthracis* are common among other bacilli, isolating *B. anthracis*-specific antisera is challenging. Since polyclonal antisera will most probably react with other closely related spores, mono-specific antibodies must be used. These antibodies can be incorporated into simple lateral flow type devices [70, 71] or in sophisticated robotic chemiluminescent systems usually coupled to magnetic devices [67]. These antibody-based devices are relatively less sensitive than PCR when used to detect spores; nevertheless, these tests are robust and less sensitive to contamination than the PCR based tests [67]. They are easy to use and can be easily operated by first responders. As in the case of PCR, the specificity of the antibodies is limited to the spore bank and the number of samples that were physically tested, usually no more than a few hundred. Therefore, the result of such a test is in the best case a possibility and not an indication.

It is advisable that a positive result received with one rapid test is confirmed using another type of test, applying fundamentally different technologies (PCR and immune assays, for example), and that if one result is positive and one negative, to wait for the result of the classical and definitive microbial growth-based assays [18, 30]. The relevance of a positive result in the rapid tests but a failure to grow the sample on growth media is questionable, since it indicates that the sample contains *B. anthracis* spores but they are most probably non-viable.

5.5 Decontamination and Risk Assessment

The first response to a verified spore contamination must be evacuation and quarantine of the stricken region (confirmed or suspected). This action has both short and long term implications that depend on the nature of this location. In 2001, the contaminated region was comprised of buildings and office spaces in an urban setting that were relatively easy to monitor and control (e.g., the broadcasting companies in New York and Florida or the Senate offices on Capitol Hill) [19, 29, 72]. The processing and distribution centers in Trenton, New Jersey, Washington, DC, and Wallingford, Connecticut, proved complicated [29]. Although all these

facilities were contained, trafficking of artifacts to and from these facilities was very high. The cross contamination that led to contamination of additional centers, local post offices and distributed mail, made the exact determination of the contaminated region extremely complicated [62, 73]. In a way, this type of contamination represents a case of small scale spore discharge in a major transportation terminal such as an airport or train and bus central stations. It was estimated that the letters sent to Senators Tom Daschle and Patrick Leahy contained only 1–2 g of spore powder in sealed envelopes [25, 74]. Since most probably these envelopes were the source of contamination of the processing centers, one can only imagine the extent of cross contamination if the powder had been discharged directly into these sorters.

In 2001, the contaminated buildings were decontaminated at different times, depending on their role and the feasibility of relocating required functions to alternative locations [29]. The American Media, Inc. in Boca Raton, Florida, was the first place where *B. anthracis* spores were detected. Though the spore-containing envelope was never found, the building was extensively contaminated [19]. In this case, the building was evacuated and the workers were permanently relocated. The building was purchased by the remediating company, which planned and executed a remediation process that took four years until successful decontamination could be declared [19]. This was not the case in the other corporate and senate buildings, where permanent relocation was undesirable or impossible [19, 29]. In this case an expedited procedure was used that targeted only contaminated areas, not entire floors or even entire buildings [75]. This was generally an intensive process taking place 24 h a day, 7 days a week, lasting up to 3 months [19, 29, 75]. The contamination included safe removal of furniture and carpets for offsite fumigation followed by repetitive chlorine dioxide fumigations of the facility, until testing confirmed that the building was clean [19, 29, 75, 76].

The three US postal processing and distribution centers are good examples of a potential bio-terror dissemination of *B. anthracis* spores in a high-traffic location. In addition to the three-major locations, *B. anthracis*-positive samples were detected in 20 US postal facilities [25, 74]. The contamination in these locations was significantly lower and in some cases, when only a few positive samples were detected, a simple cleaning of the area around the suspected location or disposal of the contaminated item, was sufficient [76, 77]. The decontamination of the three major distribution centers was much more complicated and lasted for more than 2 years [19, 29]. These distribution centers were evacuated and closed, followed by intensive cycles of sampling and fumigation, performed until they could be declared clean. The decontamination process included fumigation with chlorine dioxide or paraformaldehyde of the entire facility, or the area of the sorting machines [19]. The damage of the *B. anthracis* bio-terror event of 2001 is estimated at $1 billion, a sum that includes the actual decontamination costs, contamination testing, and facility remediation [18, 19, 25]. This sum does not include the medical expenses of treating the 11 inhalational anthrax patients.

The definition of a decontaminated area as safe was and continues to be a major challenge. The human spore dose required to kill 50% of infected subjects by inhalation remains unknown [18, 78]. Since most of the available human data is

usually circumstantial, the estimation relays on extrapolations from animal model data [18, 78]. The Sverdlovsk accident demonstrated that humans are significantly less sensitive to anthrax than livestock, since anthrax-related deaths in farm animals were detected up to 50 km downwind from the discharge point whereas human deaths only occurred up to 4 km away [20, 43]. In all other types of anthrax, the spores and/or vegetative bacteria are delivered into the host through contact with skin lesions or by ingestion [2]. In these cases, the contaminating particle size is irrelevant. Inhalational anthrax was demonstrated to be a lower respiratory tract infection, requiring alveolar deposition of inhaled spores [14, 16, 17]. Therefore, the infective particle must have a diameter of 5 μm or less for effective alveolar deposition. In nonhuman primates (NHPs), increasing aerosol particle size to 10 μm resulted in an at least one order of magnitude increase in the dose required for lethality [16]. Consequently, extrapolation from animal data must take into consideration the aerosol particle size and the relative retention coefficients between the animal models and humans [6, 14, 16, 18, 50]. Surprisingly, the reported LD_{50} values of airway inoculation of spores by either aerosol or intranasal instillation are relatively similar; in the rage of 10^4 to mid-10^5 CFU in guinea pigs, rabbits, and NHPs [78, 79]. Therefore, the US Department of Defense (DOD) estimates that the LD_{50} for spore aerosols of $\leq$5 μm of particles in humans is 8×10^3–1×10^4 CFU [18]. This estimate is for an acute, single dose exposure, but what is the risk of remaining in a contaminated area, constantly inhaling low spore amounts? Repeated exposure experiments were performed in rabbits and NHPs. There was no indication of a cumulative effect of repeated low-spore dosing [50, 79]. However, these rabbit experiments used daily doses and not a continuous exposure. Continuous exposure in humans was documented in the late 1950's in goat hair-processing mills in New England, USA, where in 1957 in a New Hampshire mill a fatal case of inhalational anthrax was recorded. This case was followed by four additional cases, three of which were fatal [6, 80]. Up until then, only cutaneous cases were reported: 140 cases in 16 years in this mill and 24 cases in 10 years in a Pennsylvania mill, where no inhalational cases were reported. As part of the investigation, *B. anthracis* spores were sampled in two sections of these two mills; the carding area, which was considered heavily contaminated, and the weaving area, which was considered clean [14]. The air in these locations was analyzed on two different occasions (while the mills were active) for total spore counts (all particle diameter sizes) and the spore fraction containing particles of $\leq$5 μm in diameter. The measurements of the $\leq$5-μm particles at the Pennsylvania mill were lower than those at the New Hampshire mill. According to these measurements, inhalation of 1300 spores of all particle size, of which 510 were $\leq$5 μm in size, during an 8-h shift did not induce infection in non-immunized workers [14, 18, 79]. In addition, in 2001, serology testing of people that were considered as in risk of exposure did not find any seroconversion, implying either no infections or low non-successful infections [81].

In spite all of the above, the limit, which was first determined by the NBC management and then applied for all other facilities, was that the decontaminated locations were safe only when all the environmental samples collected in the facility were completely negative for *B. anthracis* [29]. Though this might have been a

reasonable decision in this case because the contamination was contained and in most cases the element of time and resources was not an issue, in the case of a major contamination of a central airport or train station, this standard will not hold. The same implies in the case of a major environmental contamination, such as the event in Sverdlovsk, where a massive vaccination effort of the population in risk was initiated to eliminate the possibility of secondary infections [20]. A similar protocol was applied in Russia during the summer of 2016 reindeer anthrax epidemics in Siberia, during which the population was vaccinated in parallel to livestock [82].

5.6 Forensics

Once confirmed, the forensic examination of a bio-terror event is a combination between a criminal laboratory investigation aimed at gathering criminal evidence and a biological laboratory investigation aimed at studying the infective agent. In 2001, it took nearly a year for the investigators to identify the exact location from which the anthrax letters were mailed [25]. At the FBI laboratories, the letters were analyzed for ink type, fiber, DNA, paper properties and hand writing, without any significant finding that would contribute to reveal the identity of the sender [25]. It was established that the ink on the letters from the NY post or NBC and mailed on September 18th, was different from the one used in the letters mailed on October 9th to the Capitol offices. Other than that, no significant finding was reported. Preliminary physical analysis of the spore powder included different type of microscopy: Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), light microscopy and High Resolution SEM/Energy-Dispersive X-ray microanalysis (EDX); Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) that provides information regarding the elemental composition of the spore powder; Gas Chromatography Mass Spectrometry (GC/MS) to analyze the sample for the presence of growth medium; Accelerator Mass Spectrometry to identify the relative age of the material using C^{12}/C^{14} isotope ratios and Stable Isotope Ratio to provide information regarding the possible geographic origin [25]. These analyses, according to the report of the US Department of Justice and the Committee on Review of the Scientific Approaches Used During the FBI's Investigation of the 2001 Bacillus Anthracis Mailings [74], revealed two major findings: (a) the findings are "inconsistent with weapons-grade *anthracis* produced by offensive, state-sponsored biological weapons program", which means, this was most likely a terror attack; (b) a silicon and oxygen signal that was localized to the spore cortex (inner layer of the spore) and tin and iron, which all are indicative of specific growth conditions. None of these findings could point to the exact origin of the spores or the identity of the terrorist(s) [74].

Since anthrax is an endemic zoonosis in wide parts of the world, without direct evidence of deliberate spread (i.e. spore-containing powder or a spraying device), any human case of anthrax must be quickly categorized as either a natural case or an act of terror. Epidemiological investigation can provide the first indications to the source of infection, as was extensively discussed previously. Forensics of

B. anthracis from clinical, environmental or spore-containing artifact samples (such as mail items) can indicate whether the cause of the infection is endemic and hopefully correlate the isolate to a specific source. In 2001, the investigators, by examining the spore-containing envelopes in parallel to the clinical isolates, using relatively basic genetic typing systems, succeeded to identify the bacillus strain as Ames [25, 74]. Though the Ames stain was isolated in 1981 from a dead cow in Sarita, Texas, this strain is currently used as a gold standard in the defensive program by US laboratories mainly on the mainland [74]. This finding pointed towards a laboratory within the USA as the source of the spores. To identify the source, an extensive and robust genetic analysis was needed. Basically, the same general methods that are available today were available in 2001, whereas the main difference is the timescale [74]. Whole genome sequencing, used to finally identify the genetic variations in the strain that was used to fill the envelopes and to correlate the strain to a specific laboratory, took months whereas today it would be a matter of days [83]. In 2001, since the genomic sequence of the Florida clinical isolate was almost identical to the Ames genome deposited in GenBank, efforts were directed to the spore powder [25, 74] Using classical microbiological techniques, a search was made to identify colonies with modified morphology or different sporulation efficiency in the general population that grew after plating the powder samples on agar plates [84]. Though this effort eventually enabled the identification of four unique genetic markers, the process was time-consuming and delayed the investigation [25, 74]. The 2001 investigation ended on 2008 with the self-inflicted death of the main FBI suspect, Dr. Bruce Ivins. In 2010, the US Department of Justice released an investigation summary [25], and in 2011 the scientific committee of the US National Academy of Science (NAS) that was appointed to review the scientific findings of the investigation published its review [74]. The scientific committee states that "It is not possible to reach a definitive conclusion about the origin of the *B. anthracis* in the mailings based on available scientific evidence alone".

Overall, after 600,000 FBI investigation hours, there is still debate as to whether Dr. Ivins was responsible for the attack or whether the persons responsible for this terror attack are still at large [74]. New Next Generation Sequencing (NGS) enables the sequencing of hundreds to thousand genomes in a relatively short time. Such analysis could provide a stronger lineage of the spores in the powder to a specific spore source [74], but again, it is uncertain if it could serve as the sole evidence for indictment.

5.7 Media and Public Relations

Acts of terror rely on the open media to magnify their effect by rapid and prolonged exposure of worldwide public opinion to the event. Acts of terror without a public audience, even successful ones, are typically useless to the terrorist. ISIS for example, uses internet and social media to publicly expose remote decapitations of western hostages to generate fear all over the world. Censuring news is impossible or at least highly undesirable, especially in democratic countries. The challenge is to be

able to work with the media to control and contain an event by providing accurate information on the possible risk, signs of disease, and desired course of action, including preventive measures to curtail further transmission. In Sverdlovsk, the Soviet government controlled the media and limited the release of information to the public using a "need to know" policy, excluding the vast majority of the population [20, 21]. This policy probably did not curtail rumors regarding the illnesses and deaths occurring in the region already circulating in the area, but rather would have served to increase panic. Nowadays such information control is impossible, even in most dictatorships. In 2001, the NBC offices at the Rockefeller Building in New York was one of the contaminated locations [29]. To calm and assure the safety of their employees, NBC management decided to evacuate the contaminated third floor, while extensively sampling the second and fourth floors to guarantee that they were clean [29]. Although nasal swab sampling proved ineffective, 1200 employees were tested to demonstrate to all employees that their concerns were taken seriously. In fact, not a single employee that asked to be tested was refused. The NBC management publicly announced that "even if they found one spore they would continue to decontaminate" and that they "**wanted to be able to say with a straight face to our employees that we sampled until we found no more spores**". In addition, once the decontamination effort was completed, management was present on the scene, dining with and talking to the employees to ensure that the area was safe. These actions resulted in "**no panic at any point**" according to the NBC officials [29].

Extensive nasal swabbing was performed to USPS and Capitol Hill employees who worked in the contaminated areas, with most of them starting post exposure prophylaxis (PEP). Though the US CDC estimated that 10,000 people were at risk of exposure, to ensure the public that its concerns are addressed, 30,000 people were given PEP [63]. Many more people obtained PEP directly from their physicians, often without any real indication of exposure, all to ensure that their fears were not being overlooked [63]. The success of these measurements to calm the public was moderate. During that period, the US CDC and other members of the Laboratory Response Network tested over 125,000 environmental and clinical samples [63]; in some cases the work load was so severe that a triage procedure to prioritize the samples was necessary. Laboratories had to test everything from suspicious-looking white powders (sugar for instance) to plant seeds to stuffed animals [18, 63]. Despite the assignment of specific on-site clinics to treat people from contaminated facilities, 801 patients attended one major Washington, DC, emergency department for possible exposure to *B. anthracis* during the first two weeks following the news report on the senate case [18, 85]. Therefore, the anthrax event of 2001 was often referred to as an attack with "weapon of mass disruption" [18].

The challenge in such a terror event is to interact with the public as much as possible, explaining the risk of exposure and the expected symptoms. Community physicians can be used as a first screen to identify sick people and to offer prophylaxis to relevant non-sick people. The experts that appear on news channels should be available at all times, preventing self-proclaimed "experts" from confusing the public with partial and/or non-substantiated information. Barring special cases were

contraindication exist, the authorities must never deny treatment, even if it is obvious that it is not necessary, and do its best to ensure the population that the authorities are doing everything within their power to protect the nation.

5.8 The Substance

It obvious that to cause panic, terrorists do not have to produce a single spore—any type of powder such as plaster or flour, obtained during a trip to the nearest hardware or grocery store will suffice. After 2001, encounters with any powder-containing envelope could be considered an "event", especially if addressed to an embassy, government office or a VIP [63]. A note with the word "Anthrax" will significantly increase the effect. However, the resulting news coverage on such fake attacks will be limited since nowadays first responders are equipped with rapid *B. anthracis* detection kits that usually indicate a non-event on the spot [67, 69]. Production of *B. anthracis* spores is more complicated than just sending fake letters. Looking into the challenge of spore production we must distinguish between two scenarios, large scale, high quality spore preparations and small scale, low quality, "basement" production. States or state-like entities have the time and resources to setup laboratories and facilities to grow and purify spores. They can recruit skilled workers that will produce a (semi) weapons-grade substance that then can be transferred to terrorist groups for their use. Since *B. anthracis* spores can survive for decades, another source of such high-grade substance could be from existing biological weapon stockpiles, or from nations whose biological warfare capabilities were partially dismantled followed by the nation becoming a failed, dysfunctional state. In this case, tracing a *B. anthracis* strain back to its origin will be relatively straightforward, deterring functional nations from supplying terrorists with their bio-weapon materials [84, 86]. The Japanese Aum Shinrikyo cult was able to acquire and produce *B. anthracis* spores that it then discharged as an aerosol from the top of a building [22].

Unlike some chemical or plant toxins, production of infective *B. anthracis* spores requires microbiological training. This knowledge is not specific for *B. anthracis* and laboratory training in any type of bacillus will do. The protocols are available on the internet, even from governmental sources. Usually, access to an autoclave and incubator, which could be found in any hospital or biology department in a university, will do. In this case, usually the production scale and quality will be low. Nevertheless, this preparation may be infective and, if produced correctly, sufficient to induce inhalational anthrax and a substantial number of casualties among a small population, at the designated attack site. It is almost impossible to identify the activity of a single laboratory worker using an institute's facilities to produce spores. Recently, a report from Kenya on the uncovering of a terror group that planned to launch an attack using *B. anthracis* described three of the suspects as medical interns [87]. By being a Select Agent, the availability of *B. antracis* strains is restricted in the US. However, outbreaks of anthrax in livestock are constantly reported and while in the western world human anthrax cases are rare and strictly monitored, such

monitoring is not routinely done in large parts of Asia and Africa where there are constant reports of outbreaks in wildlife, livestock and humans. These locations could be a convenient source for *B. anthracis* strains that might be used locally or shipped to a different location. Prevention of the import or production of *B. anthracis* spores therefore relies mainly on intelligence.

5.9 Conclusions

The best way of dealing with a bio-terror event is to prevent it. The efforts to identify preparations for the production of a bioterror agent by recognizing the acquisition of necessary growth media and equipment is not trivial, since none of them are uniquely used for nefarious purposes. *B. anthracis* is not the most infective bio-terror agent on the Tier 1 Select Agent list [18]. Cutaneous anthrax is easily induced but also the simplest form of anthrax to diagnose and least likely form to be lethal form. However, the durability of *B. anthracis* spores and the nonspecific symptoms of inhalational anthrax, combined with guaranteed lethality when untreated, make *B. anthracis* a prime candidate to be the used for a terrorist attack. In the case of a *B. anthracis-related* event, public awareness to the risk of mail items that contain powders is very high, as demonstrated by the many reports on hoaxes in the news. The problem is that production of spore powder is challenging and most probably beyond the reach of most local terror organizations. Most of the response protocols rely on the experience from 2001. The "problem" is that in the 2001 the "terrorist" did his best to avoid "collateral damage" by double sealing the envelopes [25]. The contamination would have been tremendous if the high-grade *B. anthracis* powder would have been mailed in regular, leaky envelopes, massively cross contaminating every mail artifact in their path. Nevertheless, high grade powders are complicated to produce requiring specific skills and expertise that usually are held only by experts of national research programs [25, 74]. Once the terror attack has been carried out, the challenge is to identify it as such. This could be achieved by environmental detection devices or, more likely, the appearance of patients seeking medical attention in local clinics or emergency wards. The first indication of a terror event in 2001 was identifying the fatal inhalation anthrax case in Florida. Six previous cutaneous cases were misdiagnosed. If the fatal case would have been misdiagnosed as bacterial meningitis (the initial diagnosis), most probably the number of casualties would have been higher. Protocols for handling bio-terror events should be applied from the moment of *B. anthracis* detection, including locating and sampling the contaminated areas, mapping and treating at-risk populations, and initiation of the decontamination process. Combined, these efforts should reduce casualties to the absolute minimum, as well as allow the restoration of normal daily life as soon as possible.

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Brucella: Potential Biothreat Agent

6

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6.1 Introduction

Brucellosis is an ancient disease, the etiologic agent being bacteria of the *Brucella* genus. While the disease has a global distribution in humans and animals, the majority of cases are reported in Mediterranean, Middle East, Central Asia, Africa, Central America, and Latin America [1, 2]. Due to the potential for misuse of these organisms, *Brucella* spp. are categorized as group B priority agents by both the Centers for Disease Control and Prevention (CDC) and National Institute of Allergy and Infectious Disease (NIAID) in the USA. The agent is also included on the lists of potential biological agents of weapons by the World Health Organization (WHO), the Biological and Toxin Weapons Convention (BTWC), and the North Atlantic Treaty Organization (NATO) [3–5].

The perceived threat of biological agents altered drastically after the deliberate release of anthrax in the USA through postal service in 2001 for both the public and scientific communities. Trepidation surrounding biothreats has been heightening for a number of reasons. There are numerous ongoing conflicts around the world, such as those in North Africa, the Middle East, and Afghanistan, and many illegal rebel organizations (including ethnic, separatists, leftist, and religious terrorist groups) are currently very active worldwide. A huge number of people have been displaced from

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their homes, forced to migrate to neighboring countries, or become refugees because of ongoing civil war, conflict, and/or terrorist activities. The Syrian civil war is widely accepted as one of the worst humanitarian disasters since World War II [6]. On August 21, 2013, it was reported that sarin gas was dispersed in Syria, with more than 1400 civilians killed and thousands more affected [7]. International media reports suggest that chemical agents such as sarin gas or mustard gas have been used four times since the outbreak of civil war in 2011. It has been rumored that the biological agents *Bacillus anthracis* or variola (smallpox) virus could be used as a biological agent by the terrorist groups in the Middle East.

Brucella bacteria are easily obtainable from all routine diagnostic hospital laboratories. The agent is moderately easy to disseminate and results in moderate morbidity and low mortality rates. However, *Brucella* infections lead to huge economic losses in endemic countries, and there is still no available licensed human *Brucella* vaccine. Our commercial food chain is a particular area of vulnerability. The commercial food chain is highly complex involving a wide range of global producer and distributers. The intentional contamination of food supplies with *Brucella* is likely to result in major public anxiety and fear. Furthermore, *Brucella* spp. are highly infectious via the aerosol route; thus it could easily be misused as an agent for biological warfare. The global risk of biological attack increases annually due to migration, growing numbers of refugees, global travel and trade, terrorist interest in weapons of mass destruction, and advances in technology that have reduced the skill and technological resources required to manipulated pathogens [4, 8–10].

6.2 Microbiological Characteristics

Brucella species are aerobic, gram-negative intracellular coccobacilli or short rods (0.5–0.7 μm in diameter and 0.6–1.5 μm in length). The genus *Brucella* is a member of the family *Brucellaceae*. Currently, 11 recognized species have been reported: 6 terrestrial, 3 marine, and 2 proposed species. Up to 1985, the genus of *Brucella* was classified into six species: *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. ovis*, and *B. neotomae*. These are referred to as the as the six classical species and are all genetically related. *B. melitensis* and *B. suis* are generally more pathogenic in humans than *B. abortus* and *B. canis.* The species are further subdivided into biotypes, three have been defined for *B. melitensis*, seven for *B. abortus*, and five for *B. suis* [11–14]. In addition to the six classical species, five new species of *Brucella* have been identified: *B. ceti*, *B. pinnipedialis*, *B. microti*, *B. inopinata*, and *B. papionis.*

Brucella spp. are non-encapsulated and do not produce spores or flagella. They are readily grown on the common media used in microbiology laboratories. Optimal culture occurs on trypticase soy agar, *Brucella* agar, and serum dextrose agar using the classic biphasic culture (solid and liquid), blood culture technique, at a temperature of 35–37 °C with a pH of 6.6–7.4. While conventional culture requires a prolonged incubation of between 6 and 27 days, this is shortened by the use of

automated blood culture systems (up to 5 days incubation). Some biovars of *B. abortus* and *B. suis* require supplementary carbon dioxide, especially for primary isolation. When the bacteria are grown on blood agar, colonies are usually seen 0.5–1.0 mm in diameter, raised, and convex [1, 11].

The bacteria are able to persist in the environment for a long time (weeks or months) depending on the number of bacteria, sunlight, temperature, humidity, pH, nutrients, and presence of bacterial contents (Table 6.1). It is also known that the bacteria maintain their virulence in dry preparations for years [1, 11, 15, 16]. They are, however, sensitive to most commonly used disinfectants, pasteurization, heating, and ionizing radiation.

6.3 Brucella as an Agent of Biothreat

Biological agents have already been used for the purpose of "biological warfare," "bioterrorism," or "biocrime." Although no accepted definition exists, we propose the following definition: Biological warfare is the use of weaponized biological agents by a government against military targets. Bioterrorism is the threat or use of biological agents/toxins by individuals or groups to further their aims (political, religious, ethnical, or other ideological objectives). The use of biological agents for the purpose of murder, revenge, or exaction is called as "biocrime." A terrorist attack can be distinguished from criminal attack by their main objectives [3, 17]. The organisms listed by the WHO or CDC as potential biological agents are subdivided into three categories: antipersonnel, anti-animals, and anti-plants. As *Brucella* spp. are capable of causing disease in both humans and animals, there is the potential for them to be used to target both human populations and livestock [3, 5, 10].

During World War I, between 1932 and 1945, Germany initiated a biological warfare program with the intention to infect livestock and contaminate animal feed predominantly with *Bacillus anthracis* and *Burkholderia mallei.* During World War II, German scientists conducted biological weapons research on prisoners in Nazi concentration camps, testing live preparations of *Rickettsia*, hepatitis A virus, and *Plasmodium* spp. In published reports, it is not clear whether or not *Brucella* species were used [4, 17, 18]. Japan also conducted an extensive biological weapon program during World War II in Manchuria (unit 731 and 100). Experiments with various agents were carried out on prisoners of war, at least 10,000 died due to infections as part of this program [3, 18]. During the attack on Changteh in 1941, there were 1700 deaths and approximately 10,000 casualties due to biologicals among the Japanese troops; most cases were due to cholera. Although the Japanese biowarfare research program continued until the end of the World War II, field trials were terminated in 1942 [18].

In 1942, the USA had initiated an offensive biological program which was expanded during the Korean War (between 1950 and 1953). During the 1960s, the US military developed range of biological weapons, including various bacterial agents and the *Brucella* spp. During this period, *B. suis* was weaponized and formulated to maintain long-term stability and viability. Between 1944 and 1945,

Table 6.1 Survival periods of *Brucella* bacteria in various environments and substrates

Substrates or environment	Temperature and environment	Surviving time
Brucella melitensis		
Broth	pH > 5.5	>4 weeks
Broth	pH 5	<3 weeks
Broth	pH 4	1 day
Broth	pH < 4	<1 day
Soft cheese	37 °C	48–72 h
Yogurt	37 °C	48–72 h
Yogurt	5 °C, fat rate; 10%, 1.5%, and 3.5% pH 4.2–4.3	2, 3, and 5 days
Buffalo's yogurt	4 °C	20 days
Cream	4 °C	>4–17 weeks
Milk	37 °C	7–24 h
UHT milk	20 °C	>12 weeks
Dust	Depends on ambient humidity	15–40 days
B. abortus		
Solid surfaces	<31 °C, sunlight	4–5 h
Tap water	−4 °C	114 days
Lake water	37 °C, pH 7.5	>1 day
Lake water	8 °C, pH 6.5	>57 days
Soil dried	≈20 °C	<4 days
Soil wet	<10 °C	66 days
Manure	Summer	1 day
Manure	Winter	53 days
Farm slurry animal waste	Ambient temperature tank	7 weeks
Farm slurry animal waste	12 °C	>8 months
Cream	2–4 °C	>6–16 weeks
UHT milk	20 °C	>87 days
Sterilized milk	Room temperature	10 months
Buffalo's yogurt	4 °C	30 days
Yogurt	5 °C, fat rate; 10%, 1.5%, and 3.5% pH 4.2–4.3	2, 3, and 5 days
Brucella spp.[a]		
Water	20 °C	2.5 months
Still mineral water	20 °C	63 days
Raw milk	8 °C	2 days
Ice cream	4 °C	30 days
Cheese	Room temperature	3–12 weeks
Butter	8 °C	142 days
Meat	Frozen meat	<3 weeks

Summarized from the references [1, 13, 15, 16]
[a]Not given the species

B. suis was loaded into bombs, and field trials were carried out to test its efficacy against animal targets. Approximately 10 years later, human experimentation on military and civilian volunteers was conducted, using spherical aerosolization chambers in volunteers who were exposed to microorganisms when biological munition was exploded. By 1969, the USA announced that the offensive *Brucella* program had been terminated and all biological munitions were destroyed. They also state that the munitions developed were never used in conflict [17, 18].

The former Soviet Union had an extensive offensive biological weapons program. *Brucella* was one of the agents which they were working on. Ken Alibek, former deputy director who moved to the USA in 1992, stated that antibiotic-resistant strains of *Brucella* were developed and weaponized both in dry and liquid forms with production capability ranging up to hundred tons [4]. He also described a sophisticated system that had been constructed for bacterial delivery which had been extensive field tested in the Aral Sea [4]. By the end of twentieth century, interest in *Brucella* had waned, and the organism was replaced by *Burkholderia pseudomallei* in the biological weapon program [4, 18].

Several microbiological characteristics of the *Brucella* species make it tractable as a potential agent in bioterrorism or bio-war. These bacteria, particularly *B. melitensis* and *B. suis*, are highly infectious through the aerosol route, and the infectious dose for humans is relatively low, approximately ten to a hundred microorganisms. The organisms can enter the body through the respiratory mucosa and gastrointestinal tract, genital mucosa, conjunctivae, minor skin lesions, or abraded skin. The incubation period ranges from up to 1 week to several months. The infection may mimic infectious or noninfectious diseases. In humans, brucellosis is a debilitating and prolonged disease with acute, subacute, or chronic forms. The disease requires long-term antibiotic therapy, and there are only a limited number of antibiotic currently being used for treatment [4, 9].

Computer modeling suggests that following an aerosol attack with *B. melitensis*, the epidemic curve, by days after exposure, shows that 4% of cases would occur within 0–7 days, 6% in 8–14 days, 14% in 15–28 days, 40% in 29–56 days, 26% in 57–112 days, and 10% in more than 113 days. It was calculated that the economic cost of such an attack would be $477.7 million per 100,000 exposed people [19]. It is estimated that the release of 50 kg of *B. suis* from a plane along a 2 km line at a distance of 10 km upwind of a city of 500,000 people would result in the infection of 125,000 people and 500 deaths [18].

Another route of biological attack using *Brucella* is the deliberate contamination of commercial food products or animal feeds. This contamination could potentially occur during production, packing, storage, transportation, or delivery. Records of intentional or malicious contamination in the food supply chain between 1950 and 2008 were collected and analyzed; during this period, 464 events were recorded resulting in a total of 4187 deaths and 19,545 injuries [20]. It was reported that 12 of these events and 190 deaths are attributable to biological agents [20]. According to a report entitled "Chronology of Chemical and Biological Incidents Targeting the Food Industry," more than $100 million in lost income had been recorded between 1946 and 2006 [21].

To date, *Brucella* species have not been used against either civilians or military targets. However, this does not mitigate the potential threat of the intentional use of *Brucella* spp. in both endemic and non-endemic countries (particularly in Western countries). Given its zoonotic nature, an attack with *Brucella* spp. could lead to severe disease outbreaks in either human population or farm animals. There is currently rising concern around the danger of agroterrorism, targeting farm animals such as sheep, cattle, swine, and fish, processed food, and food storage facilities.

6.4 Brucellosis as a Zoonotic Disease

Brucellosis is a zoonotic disease, with the source of natural human infection being infected animals. Natural reservoirs of *Brucella* spp. are sheep and goats (*B. melitensis*), cattle (*B. abortus*), swine (*B. suis*), and dogs (*B. canis*). In Table 6.2, the reservoir host and potential human pathogenicity of *Brucella* spp. is outlined. The more recently described species of *Brucella* spp. were identified from wildlife hosts which include rodents, marine mammals, and baboons. Some of these species have not been widely identified in human infection; thus their infectivity and virulence is not completely understood [11, 12].

Generally, the animal reservoirs of *Brucella* spp. (Table 6.2) are asymptomatic carriers. Although subacute or chronic presentation of the disease may also be seen in infected animals, within the host, the bacteria target organs and tissues, in particular the reproductive system which includes the placenta, mammary glands, testis, and epididymis. *Brucella* infection results in placentitis and miscarriage during the last trimester of pregnancy. Epididymitis and orchitis are seen in the male. There are no specific clinical indications of brucellosis in animals, and

Table 6.2 The host preference of *Brucella* species and pathogenicity for humans

Species	Reservoir	Pathogenicity for humans	Human cases (worldwide)
B. melitensis	Sheep, goat, camel	High	++++
B. abortus	Cattle, buffalo, yaks, bison	High	+++
B. suis	Swine	High	++
B. canis	Dog	Moderate	Rare
B. ovis	Ram	No	No reported cases
B. neotomae	Desert and wood rats	No	No reported cases
B. ceti	Dolphin, porpoise, whale	Mild	Few cases
B. pinnipedialis	Seal	Mild	Few cases
B. microti	Vole, fox, soil	Unknown	No reported cases
B. inopinata	Unknown	Mild	Few cases
Brucella papionis sp. *nov*	Baboons	Unknown	No reported cases

Summarized from the references [11, 12, 14, 22]

diagnosis is based on the isolation of bacteria, the detection of bacterial antigens in clinical samples, or the demonstration of a specific antibody response. Transmission can occur directly between animals, which can result in miscarriage, or alternatively during mating from genital secretions or semen during or to offspring via milk. In domestic animals, infection can occur if the barn, pasture, animal feed, and/or water sources have been contaminated. *Brucella* infection leads to abortion, stillbirths, decreased fertility, and low milk production in livestock. Therefore, infection results in economic losses and can pose a serious public health threat in endemic countries [12, 14, 22].

Transmission to humans occurs through direct contact with infected animals and/or their excretions (urine, semen, and mammary fluid, genital secretions), contaminated blood or carcasses, or dairy products (milk, fresh cheese, cream, butter). Naturally occurring brucellosis is regarded as a food-borne disease, an occupational infection, or rarely laboratory-acquired infection. The disease is predominantly acquired from the consumption of raw/unpasteurized milk or other unpasteurized dairy products, particularly fresh cheese. Another common source of infection is occupational contact with infected animals. Farm workers, shepherds, butchers, veterinarians, and meat-packing employees are considered to be at high risk in endemic regions [1, 12, 22]. Laboratory workers (particularly those working in hospital diagnostic laboratories in endemic countries or in reference laboratories for zoonotic disease) are also at risk of *Brucella* infection [23, 24], and accidental laboratory-acquired infection has been reported worldwide. While human-to-human transmission is rare, brucellosis resulting from sexual transmission or blood transfusions has been reported [25, 26].

Brucellosis remains one of the most common bacterial zoonotic diseases worldwide. The WHO estimates that 500,000 new human cases of *Brucella* infection occur annually [2], and infection of livestock leads to significant economic losses, particularly in developing countries [22].

6.5 Clinical Presentation of Human Brucellosis

Brucella infection in humans can occur through ingestion or inhalation, via contact of broken skin with infected animal tissue or body fluids through broken skin or eyes. After infecting the host, the bacterium penetrates mucosal barriers and enters the bloodstream, facilitating dissemination throughout the body [12]. *Brucella* spp. are intracellular bacteria which reside and multiply within mononuclear phagocytes (monocytes, macrophages, and dendritic cells); they are able to avoid the host's intracellular killing [27]. The bacteria spread within the phagocytic cells to the reticuloendothelial system (RES) (localizing mainly at the joints), the central nervous system (CNS), the cardiovascular system (CVS), the respiratory system, and the genitourinary tract. The incubation period of disease varies depending up the virulence of strain, the route of entry, and the infectious dose. It is often difficult to determine precisely when infection has occurred as the incubation period while using 1–4 weeks can be up to several months (Table 6.3).

Table 6.3 Clinical manifestations of patients reported from the studies published after 2000

Clinical manifestations	Interval for the percentages
Fever	55–100
Malaise	68–90
Arthralgia	66–87
Sweating	19–96
Myalgia	36–49
Back pain	6–58
Nausea/Vomiting	21–30
Abdominal pain	6–28
Hepatomegaly	6–50
Splenomegaly	7–60
Osteoarticular involvement	19–54
Cardiovascular involvement	0.4–1.8

Summarized from the references [28–35]

Brucellosis is a systemic disease which affects various organs or body systems. The disease generally presents with intermittent fever, chills, arthralgia, myalgia, and malaise. Although most commonly a systemic infection, brucellosis may also cause a localized infection involving specific organ systems such as the skeleton system, central nerve system, heart, liver, and lungs. It is also associated with focal abscess formation particularly in RES and the skeleton system. The localized form of brucellosis occurs with untreated acute or chronic disease. Focal infection occurs in approximately 30% of cases.

The symptoms of brucellosis are similar, regardless of the bacterial species involved. However, the severity of these symptoms varies; *B. melitensis* and *B. suis* cause severe infection, while *B. abortus* is associated with a greater proportion of subclinical cases, and *B. canis* infection usually causes only a mild disease [15].

Although there is no clinical experience with intentional released brucellosis, both naturally occurring and intentional released brucellosis are likely to have a similar presentation. Thus, the clinical symptoms and laboratory findings of naturally acquired brucellosis may be considered to be representative for brucellosis due to intentional release.

Cases of brucellosis are arbitrarily classified into clinical types based on the duration of symptoms. The disease is classified as “acute” when there has been less than an 8-week duration. The disease is deemed “subacute” from 8 to 52 weeks and “chronic” beyond 52 weeks [16].

Approximately 50% of patients develop acute illness; they present with a range of non-specific symptoms which include fever (over 38.5 °C in 85% of patients, with an intermittent pattern), night sweats, weakness, fatigue, malaise, headache, nausea, vomiting, arthralgia, and myalgia [28]. Upon physical examination, the clinical findings are also variable and non-specific; most commonly they will include hepatomegaly, splenomegaly, and osteoarticular involvement [28]. Patient

symptoms typically resolve within 2–4 weeks, but a limited number of patients will develop chronic disease or have relapses.

If there is a reoccurrence of disease, 3–6 months after completion of therapy, this is termed relapsing disease, and this occurs in 5–30% of patients. Relapsing brucellosis tends to be a milder form of disease than the initial attack [36]. While antibiotic resistance is currently not a significant issue in the treatment of brucellosis, relapsing diseases is often associated with the use of inappropriate antibiotic treatment of the initial disease [37, 38].

Subclinical cases of brucellosis are usually asymptomatic; they are characterized by positive, low titer, serology, and negative bacterial cultures. The subclinical form of the disease frequently occurs in abattoir workers, farmers, and veterinarians in endemic areas [16].

The chronic form of brucellosis is usually associated with undiagnosed and untreated disease. It typically has a febrile pattern and is mainly characterized by fatigue, depression, myalgia, and arthralgia. This clinic form resembles "chronic fatigue syndrome." It generally occurs in older individuals (over 30 years old) and rarely occurs in children. In chronic brucellosis, localized disease usually manifests as spondylitis, hepatitis, epididymitis, or endocarditis [39].

A meta-analysis of clinical manifestations of brucellosis provides a comprehensive evaluation of scientific literature published between 1990 and 2010 [40]. Fever was identified as the most common symptom, observed in 80% of patients regardless of age. Given this high proportion, brucellosis should be considered as a differential diagnosis for fever of unknown origin. The most common presentation of disease is musculoskeletal system involvement. Arthralgia affects 65% of patients; in contrast arthritis was reported in only 26% of patients. Arthritis generally involves large joints, with those most commonly effected, in descending order, the sacroiliac, knee, hip, vertebra, and ankle. While bursitis, tenosynovitis, and osteomyelitis have also been described, they are rarely seen. Spinal involvement is the foremost cause of the debilitating and disabling complications and is seen in 6–12% of cases. Musculoskeletal involvement is more frequent in young patients, whereas older patients are more prone to spinal involvement and complications such as paravertebral, epidural, and psoas abscess formation. The lumbar region was the most frequently involved, but it is known that the disease can affect the entire vertebral column [41]. Prosthetic joint infection due to *Brucella* spp. is extremely rare but should be considered in endemic countries.

Involvement of the genitourinary system can present with epididymo-orchitis, cystitis, pyelonephritis, interstitial nephritis, glomerulonephritis, prostatitis, and renal abscesses. These complications occur in 2–20% of cases. Epididymo-orchitis was observed in one in ten men and thus appears to be the most affected organ [40].

Neurobrucellosis is seen in 2–7% of the cases, the manifestations range from headache, alterations in behavior, and confusion to nerve deficits, acute/chronic meningitis, encephalitis, radiculitis, and myelitis. While it is not uncommon for patients to report depression, psychosis, and mental fatigue, these symptoms are greatly underestimated in the diagnosis of neurobrucellosis [42].

Pulmonary involvement in brucellosis can occur either as a result of inhalation of infectious aerosol or hematogenous spread. This presentation is rare, occurring in only 7% of patients with brucellosis [40]. Signs and symptoms of pulmonary involvement can range from mild, non-specific such as cough, mucopurulent sputum, and flu-like symptoms to sever bronchitis, interstitial pneumonitis, lobar pneumonia, lung nodules, pleural effusion, hilar lymphadenopathy, and empyema.

Gastrointestinal complaints such as dyspepsia, anorexia, and abdominal pain are frequent, occurring in up to 50% of patients with brucellosis. However, severe complications including hepatic or splenic abscess, cholecystitis, pancreatitis, ileitis, colitis, and spontaneous peritonitis are relatively uncommon. A mild to moderate increase in transaminases may be observed; 38–53% of patients have elevated baseline values of aspartate and alanine aminotransferase [43]. Mild jaundice may be observed; however, deep jaundice is seen rarely in patients with brucellosis.

Brucellosis causes hematological abnormalities; it is particularly associated with anemia and leucopenia. The disease may also cause thrombocytopenia, pancytopenia, and/or disseminated intravascular coagulation. Occasionally, brucellosis has been reported to induce severe autoimmune hemolytic anemia which is refractory to traditional corticosteroid therapy [44].

The eyes and ears can be affected with brucellosis. Ocular manifestations are most frequently seen during the chronic phase of disease, with the most common presentation being uveitis. More serious complications can also occur; these include corneal ulcers, iridocyclitis, nummular keratitis, choroiditis, optic neuritis, papilledema, and endophthalmitis. The auditory system is affected during acute brucellosis, and all diagnosed patients should be evaluated for hearing loss [45].

Cutaneous manifestations of disease are usually non-specific and only occur in 1–14% of patients with brucellosis. These can include macular or maculopapular rash, scarlatiniform, papulonodular, and erythema nodosum-like eruptions, ulcerations, petechiae, purpura, granulomatous vasculitis, and abscesses [46].

Although brucellosis is not, in itself, a fatal disease, some of the complications associated with the disease may be lethal. The leading cause of brucellosis-related deaths is cardiac and CNS complications. The incidence of endocarditis, myocarditis, pericarditis, endarteritis, thrombophlebitis, and/or mycotic aneurysm of the aorta or ventricles is low; they have been reported to occur in only 1% of cases. Recent advances in surgery, combined with effective medical treatments, have proven successful in preventing death due to endocarditis [47].

Brucellosis can be a severe, debilitating, and sometimes chronic disease with the potential to affect a variety of systems within the body. The mortality rate associated with brucellosis is as low as 2%, as appropriate treatment generally results in complete recovery without complications. Due to the non-specificity of the clinical features of brucellosis, the disease can imitate a number of syndromes and, thus, has been labeled “mimicking disease.” Infectious disease such as tuberculosis, malaria, typhoid fever, and infectious mononucleosis or other noninfectious diseases such as chronic fatigue syndrome, collagen vascular diseases, autoimmune diseases, and tumors should all be considered in differential diagnosis of brucellosis.

6.6 Diagnosis and Treatment of Brucellosis

As the clinical picture of brucellosis is non-specific in humans, diagnosis needs to be supported by medical history, physical examination, and appropriate laboratory tests. Inquiries should be made about potential occupational exposure, travel to an enzootic region, and consumption of unpasteurized/raw milk and dairy products while taking the medical history.

The gold standard for diagnosis of brucellosis is isolation of the bacteria from either blood cultures or other tissues. *Brucella* spp. can be isolated from the bone marrow, tissues (liver, spleen), cerebrospinal fluid (CSF), synovial fluid, etc. A prolonged incubation is required as *Brucella* spp. are slow-growing bacteria; however, automatized blood culture systems, which are now routinely used in most clinical laboratories, allow for the detection of bacteria within 1 week [48]. The sensitivity of the detection in blood cultures ranges from 50 to 90%; this is dependent on several factors including the stage of the disease, the culture medium utilized, and previous antibiotic usage. Identification of *Brucella* spp. and antibiotic susceptibility testing requires the use of biosafety level-3 (BSL-3) protocols due to the high risk of laboratory-acquired infections. Species-level identification, which requires detailed phenotypic or molecular assays, while essential for epidemiological studies, is not required for the initiation of therapy [49].

Brucellosis diagnosis is predominantly based on serology due to the low sensitivity of the culture. A variety of serological tests have been devised over the past 100 years beginning with a simple agglutination test. A range of tests are routinely used for the diagnosis of the disease; these include Rose Bengal plate tests (RBPT), serum agglutination tests (SAT), complement fixation tests (CFT), and an enzyme-linked immunosorbent assays (ELISA) [1, 42].

The RBPT is performed using a suspension of *B. abortus* colored with Rose Bengal stain. It is a simple and rapid slide-type agglutination test based on the reactivity of antibodies against smooth lipopolysaccharide (S-LPS). This is the preferred screening test as it has a high sensitivity of 93%. The limitation of this test is that there is a much lower sensitivity in chronic cases and reduced specificity in endemic regions. As a result of these limitations, the WHO guidelines recommend the confirmation of positive samples using SAT [1, 42].

The gold standard for serological diagnosis of brucellosis is SAT; this assay is also based on the detection of antibodies against S-LPS. The test is performed in tubes, by serially (doubling) diluting sera which reacts with a constant amount of *Brucella* antigen. The visible agglutination titers reflect the concentration of antibodies in the serum, usually ranging from 1:20 to 1:1280. Either an elevated SAT titer of $\geq$1:160 or demonstration of a fourfold increase from acute to convalescent titers is considered diagnostic. In order to reduce the incidence of false positives, in endemic areas, the recommended cutoff is $\geq$1:320. The presence of high non-agglutinating IgG defined as "blocking antibodies" may result in false-negative results in SAT. It is important to note that active brucellosis cannot be excluded in patients with SAT titers lower than 1:160. During the early stages of infection, the titer may be below the cutoff; therefore repeat testing may be required

[1, 50]. Lower SAT titers may also be seen in chronic and relapsing cases, and therefore antiglobulin (Coombs) test may be more appropriate for diagnostic confirmation of chronic and relapsing cases. In addition to these technical limitations, SAT is time and labor intensive.

The use of ELISA allows rapid, sensitive, and reliable diagnosis of brucellosis [51]. Both IgM- and IgG-specific antibody detection by ELISA have been shown to have a good concordance with SAT and Coombs tests and are more sensitive in chronic cases [52, 53]. In endemic areas ELISA is a recommended over conventional agglutination [52]. There are however conflicting views, with some studies suggesting that ELISA is less sensitive in the detection of anti-*Brucella* antibodies than more conventional serological tests [54].

A newer serological test is Brucellacapt, which is based on the immunocapture-agglutination of total anti-*Brucella* antibodies [55]. This assay shows a high sensitivity and specificity in the diagnosis of human brucellosis, not only in the first stages of the disease but also in cases with long evolution and in relapses and reinfections. A decrease in specific antibody titers following successful treatment and clinical cure is more pronounced and rapid in Brucellacapt than SAT and Coombs test. Therefore, Brucellacapt titers can be considered to be a good marker of infection, particularly when used during patient follow-up [50].

Brucella DNA can be detected using polymerase chain reaction (PCR) assays in either cultures or clinical specimens. PCR has been proven to be more sensitive than blood culture and more specific than serologic tests in both acute and chronic brucelloses. Working with highly infectious live cultures carries a risk of laboratory infection which is greatly reduced when working with DNA [56].

While complete blood count, erythrocyte sedimentation rate, C-reactive protein, and liver function tests are not specific for the diagnosis, they are useful in the diagnosis and monitoring of the disease. The sensitivity and specificity of brucellosis diagnosis are improved using a combination of two or more diagnostic tests and compatible clinical symptoms [1, 16].

The objective of the antimicrobial therapy in brucellosis is to reduce disease symptoms, shorten the duration of the symptomatic period, and reduce or prevent complications or relapses. Given that *Brucella* spp. are intracellular microorganism, antibiotics capable of reaching a high intracellular concentration must be used. Prolonged treatment with a combination of two or more drugs is recommended in order to prevent relapse [1, 11, 16].

The World Health Organization (WHO) recommends an antibiotic regimen of oral doxycycline 100 mg twice a day for 6 weeks plus oral rifampicin 600–900 mg daily for 6 weeks or streptomycin 1 g intramuscularly daily for 2–3 weeks for the treatment of uncomplicated brucellosis [1]. A meta-analysis of clinical trials published between 1985 and 2012 found that this is the most widely used treatment regimen [36]. There are, however, alternative treatment options. A doxycycline-rifampicin regimen has the advantage of oral administration, while a regimen which combines doxycycline and streptomycin has been shown to be superior, both in terms of treatment failure and relapse rates [57]. Several studies have reported the

efficacy of other alternative combinations: quinolones and rifampicin, co-trimoxazole and rifampicin, and triple regimens with doxycycline, rifampicin, and aminoglycoside [58]. The combination of co-trimoxazole and rifampicin is particularly recommended for children and pregnant women where tetracyclines are contraindicated. Monotherapy and short course of therapies (<6 weeks) are not acceptable treatment strategies for brucellosis [1].

There is no recommended treatment regimen for complicated brucellosis. For endocarditis, spondylitis, or meningitis, the agents of choice are similar. Triple therapy regimens including the combination of aminoglycosides plus doxycycline and rifampicin are considered as the first line as they offer good efficacy and low rates of treatment failure and relapse. The duration of therapy for complicated cases should be prolonged to more than 8 weeks [58].

Brucellar endocarditis is a rare complication with high mortality. The optimal antibiotic regimen and duration of therapy remain unsolved. Many authors have reported satisfactory results with perioperative antibiotic therapy and surgical treatment (prosthetic valve replacement) [47, 59].

Spinal brucellosis is the leading cause of debilitating and disabling complications. Spondylitis may extend to neighboring vertebrae. The paravertebral and epidural spaces present with abscess formation which requires a longer duration of antibiotics, occasionally combined with surgery. Surgical interventions are recommended as the last resort when there are persistent systemic symptoms despite adequate antimicrobial therapy, vertebral collapse, or septal abscess [60].

The WHO-recommended treatment of neurobrucellosis is the standard regimen of doxycycline plus streptomycin, with the addition of rifampin or co-trimoxazole. A prolonged duration of the treatment is also suggested, with a minimum duration of 6–8 weeks, with possible further extension depending on the clinical response [1]. In a multicenter study, which included 215 adult patients with neurobrucellosis, the average duration of treatment was about 4.5–6.5 months. This study also presents data supporting the use a month of parenteral ceftriaxone treatment in combination with doxycycline and rifampin. They found that ceftriaxone-based regimens provided significantly shorter duration of therapy than oral treatment [61].

Even with the use of recommended antimicrobial regimens, therapeutic failure and relapse occur in 5–30% of patients with brucellosis; this is usually associated with shorter duration of treatment or ineffective antibiotic regimens [37]. Resistance to antimicrobial drugs particularly for first-line regimens is unusual. To date, only increases in the MICs of ceftriaxone and streptomycin have been reported in Turkey [62]. Relapsing cases of brucellosis have not been shown to be related to drug resistance.

Brucellosis has a widespread geographic distribution; however it mainly affects developing countries. In order to prevent disease, it is crucial to identify simple, inexpensive, efficacious treatments and design effective control programs.

6.7 Biotechnology Applications to Detect and Identify *Brucella* Species

Biological weapons are a serious global concern [9, 63, 64]. Biotechnological advancements can be misused for the development of antibiotic- and vaccine-resistant, undetectable, more stable, easier-to-handle, and lethal biological agents which could be used in a bioterrorist attack. If a bioterrorism outbreak were to occur, clinicians, pathologists, and microbiologist's first aim would be to identify the causative agent. It may, however, not be an easy to accurately detect the microorganism due to applications of intricate genetic engineering strategies. Therefore rapid and sensitive detection is likely to require multiple methods from a variety of specimen types to facilitate the correct identification of bacteria causing the epidemic. Currently, each bacterial detection method has its own pitfalls and usually requires additional tests to confirm the results.

Various biotechnological tools can be used to detect and identify *Brucella* spp. Diagnosis of *Brucella* in samples generally relies on culture-based methods and serologic tests. Sensitive culturing of bacteria is dependent on there being sufficient numbers of viable *Brucella* in the sample. After a positive isolation of *Brucella* spp. is achieved, biotyping, serotyping, phage typing, nuclear sequencing, restriction endonuclease fragmenting, and hybridization can be used for detailed characterization of the *Brucella* species. Failure to isolate *Brucella* does not necessarily rule it out as the causative agent. Another frequently used diagnostic relies on serologic tests, which are mainly based on the detection of antibodies which are produced following infection with *Brucella* spp. Both validated and in-house agglutination assays, precipitation tests, and Western blotting tests are used for serologic detection of *Brucella* spp. in centers worldwide. Antigens from S-LPS obtained from *B. melitensis* and *B. abortus* are generally for the serological diagnosis of *Brucella* spp. Due to the existence of *B. canis* and *B. ovis* as rough colony forms, detection of antibodies for these species is only achievable using major outer membrane protein antigens. The requirement for multiple testing for accurate assignment of *Brucella* species is a limitation of serology tests. There is a need for identification of novel target antigens to be used in these tests, for example, there are currently no specific serologic for the detection of *B. melitensis* infection in small ruminants [65]. Another limitation of serologic testing is the lack of standardized reference antigen, resulting in variations in the test results [42]. To accurately differentiate species and biovars, serologic testing is used in combination with PCR-based techniques, such as enterobacterial repetitive intergenic consensus sequence PCR, repetitive intergenic palindromic sequence PCR, amplified fragment length polymorphism analysis, monolocus sequence analysis, and multi-locus variable-number tandem repeat analysis [9, 42]. The sensitivity and specificity of these techniques for accurate detection of *Brucella* spp. are dependent on the laboratory conditions and a highly skilled technical personnel existence; there is a requirement for the development of robust, standardized, and validated methods.

While isolation of *Brucella* bacteria is considered the decisive method of diagnosis for brucellosis, due to the difficulties associated with this technique and

serological testing, new efforts to standardize and validate PCR-based diagnosis techniques are underway. PCR-based technologies offer sensitive and reliable detection of the genus. The development and validation of these tools for routine diagnosis will also eliminate issues associated with contamination with other bacteria, most commonly *Yersinia*. Hundreds of PCR-based methods have been developed for the detection and typing of *Brucella* spp. directly from milk, whole blood, serum, semen, body fluids, and tissues from neonates of aborted fetus [9, 42, 56]. They all involve the extraction of DNA using available commercial kits. Depending on the source of the sample, the efficiency of the kits' DNA isolation capacity will vary [56]. The extraction of DNA from blood can be problematic due to the presence of inhibitors; this necessitates repeat washing of the blood with either water or lysis buffer, removing contaminating hemoglobin. Single pairs of PCR primers to identify *Brucella* spp. at the genus-specific level are used for testing human blood samples; however higher sensitivity is achieved when targeting multiple genes (especially the combinations of primers targeting *bcsp31*, *omp2a*, *omp2b* genes) in a single PCR reaction [56, 66]. Additional improvements have facilitated the use of multiplex and real-time PCR assays [66], both of which have been shown to be highly effective for the detect *Brucella* spp. at a biovar level [56]. There can however be some misleading results, for example, discerning *B. suis* biotype 4 from *B. canis* at the biovar level, due to similarities observed in their PCR patterns [67]. Molecular methods are faster and more sensitive than traditional methods; despite this, the routine application of these tests for the diagnosis of *Brucella* spp. is currently limited. Validation of these tests is necessary in order to meet the quality control and assurance criteria for diagnose of *Brucella* infection in clinical samples, before they are used in routine laboratories. Additionally, since these PCR-based methods rely solely on the current genomic knowledge of *Brucella*, these methods should be updated as variations arise in *Brucella* genome.

While improvement in both serologic and PCR-based methods is underway, there are efforts to find alternative routes to diagnose a quantitative brucellosis using biosensors or *Brucella*-specific nanobodies. These assays have the potential to offer rapid, inexpensive, and easy-to-use methodologies for the detection of *Brucella* bacteria in the environment or clinical samples [9, 68–70]. Biosensor-based detection technologies of bacteria quantify the signal produced after a biological response is converted to electrical signal. Most of the biosensors are based on labeling techniques, where the target molecules get labeled either before interaction or after binding of the target on the sensing surface. Due to the long time scales and high costs associated with the development of labeling-based, optical, label-free, biosensors are being investigated. Optical biosensors also offer the potential for real-time detection. Various types of biosensors have been designed for *Brucella* spp. detection, some of which allowed very specific recognition [9, 68–70]. Recently, two nanoscale biosensors were designed which utilize gold nanoparticles and oligonucleotide probes to directly visualize *Brucella* spp.; these allow detection of *Brucella* spp. at pg/μL concentrations [69]. The same researchers have also designed a label-free DNA hybridization-based electrochemical geno-sensor on palladium nanoparticles which acts as a transducer allowing for the sensitive quantification

and detection of *Brucella* species [68]. A surface plasmon resonance immunobiosensor has been developed which targets DNA fragments of *B. melitensis* using two different probes covalently attached to different 4-MBA/Au SPR chips [70]. This SPR-based biosensor allows label-free nanomolar range detection of *B. melitensis*; this holds promise as a rapid and sensitive detection technique in pathology laboratories. Another new detection strategy is based on nanobodies, which are single-domain camelid-derived antibody fragments that have been genetically engineered and are highly soluble and stable. Nanobodies are retrieved from *Brucella*-immunized camelid (NbBruc02 and 03 constructs) using phage display; this is followed by re-cloning the genes in a protein expression plasmid and subsequent purification of the nanobodies [71]. These nanobodies can detect *B. abortus* and *B. melitensis* antigens, offering the ability to differentiate the two main but highly similar species [71].

6.8 Control and Prevention

Public health preparedness, early stage responses, and counter measurements are very important for the prevention of intentional released biological agents. Biological threat analysis and public preparedness require a multidisciplinary approach which should include law enforcement, governmental organization, and medical and scientific preparedness. Public health preparedness includes medical awareness, surveillance, laboratory skills, and diagnostic capabilities in order to strengthen our ability for identification of the potential biological agents in developing and developed countries. An effective system is required to allow the intelligence and security services, law enforcement, and health authorities to work together. Both civilians and the majority of healthcare workers have little or no knowledge of the potential illnesses caused by biological agents including *Brucella* species. They may not, therefore, suspect a deliberate released disease during the early phase of an incident. There is a need to train healthcare workers (HCWs) in the recognition and initial management of biological incidents. Education and training program must cover the characteristics of biological agents, clinical presentations, diagnosis, treatment and prophylaxis of the disease, infection control procedures for HCWs, suspected sample collection, and contaminated sample handling, as well as decontamination procedures. Rapid communication systems between governmental organizations are also required to allow the immediate sharing of information when an unusual incident is suspected [1, 5, 9, 10].

An intentional release of *Brucella* species would not cause a sudden outbreak of disease. The outbreak could induce a smooth curve, gradually increasing followed by a decrease over a period of 2–3 months [19]. Local governors and security personnel must therefore be aware of the suspected incidents in their regions. Primary care and family physicians, public health workers, emergency service physicians, infectious disease physicians, and hospital epidemiologists should be aware of clustered human cases of brucellosis. Indications of the deliberate release of *Brucella* would include large-scale outbreaks or unusual setting clusters of

brucellosis, especially where *Brucella* infection is in not endemic cases with no previous travel history to endemic regions or suspected food consumption and no history of occupational or laboratory exposure. Veterinarians and veterinarian health workers should also be aware of increasing animal cases in their regions. The source of unexpected *Brucella* infection and clustered human and animal cases must be analyzed epidemiologically. In endemic countries, it will be very difficult to differentiate naturally occurring *Brucella* infection from intentional released infection. Many physicians working in industrial countries are not familiar with clinical presentation of brucellosis. For this reason, the diagnosis of human cases may be delayed [4, 8, 9].

If an attack were to occur, appropriate environmental sampling and rapid identification of the agent released is essential to allow the appropriate preventive and medical measures to be rapidly instigated. Planned intervention should include triage of suspected or known exposed victims, protection of HCWs and other responders, prevention of public fear and panic, initiation of decontamination procedures, prophylaxis, and monitoring the outbreak. For the early detection of biothreat agents including *Brucella* spp., molecular techniques such as genetic probe assay, nucleic acid amplification, immunoassay, and enzyme inhibition using a silicon-based biosensor are now available. Some of the biosensors which have been recently developed to detect the *Brucella* spp. in the environment may also be employed [1, 4, 9, 14].

In the event of a biological attack, HCWs, technicians, and other responders should wear the N95 masks, goggles, impermeable clothing, gloves, and shoes to protect them from airborne *Brucella* infection. All victims should be evacuated from the attack area. Although *Brucella* spp. are unable to penetrate intact skin, the biological agent from human skin should be removed using water or soap and water; the clothes from victims should be disposed of in order to minimize the risk of infection by accidental conjunctiva and other mucosal inoculation or ingestion of viable bacteria. All contaminated victim clothes should be burned or decontaminated by effective disinfectants [1, 5, 9, 72]. For hospitalized patients, patient isolation is not required because of the low risk of human-to-human transmission [1].

Contaminated foods should be destroyed, by the trained individuals, in protected areas. The *Brucella* bacteria can survive in the environment for varying periods (Table 6.1). Buildings can be decontaminated using chlorine-based liquid sprays, formaldehyde steam produced by heating paraformaldehyde, or other disinfecting fumigants. In limited areas, 3% phenol or 10% hypochlorite solution may be applied by a trained person wearing a protective mask, goggles, gloves, and gown. Currently, it is extremely difficult to certify that a building is clean after decontamination due to an intentional release of a biological agent [1, 5].

Vaccination is very important components for the prevention of infection in individuals' exposure to released *Brucella*. Although there is no licensed human *Brucella* vaccine, the live human vaccines *B. abortus* strain 19-BA and *B. melitensis* strain 104 M have been used in the former Soviet Union and China, respectively [22, 73]. Human vaccine studies are under development; however they have only shown limited efficacy and induce serious medical reactions. Subunit vaccine studies

Table 6.4 Recommendations for post exposure prophylaxis of brucellosis

	Regimen (administration route and daily dose)
Adults	Doxycycline: 100 mg bid per os and rifampicin: 600–900 mg per os once daily
Pregnant women for breastfeeding women: cessation of breastfeeding is recommended	Doxycycline: 100 mg bid per oral and rifampicin: 600–900 mg per oral once daily
Children >8 years	>45 kg: adult dose <45 kg: 2.2 mg/kg per oral twice daily and rifampicin: 10–15 mg/kg per oral in 1 or 2 doses daily
Children <8 years	Trimethoprim (6–8 mg/kg/day) and sulfamethoxazole (30–40 mg/kg/day) per oral in 1 or 2 divided doses and rifampicin: 10–15 mg/kg per os in 1 or 2 doses daily
Recommended duration of prophylaxis	3–6 weeks

Summarized from the references [1, 4, 8, 9, 72]

are, however, showing promise for successful future vaccine development [73, 74]. Most veterinary vaccines are based on live-attenuated strains; they have been successful in the control of livestock infections. The most commonly used veterinary vaccines against *Brucella* infection are *B. abortus* strain 19 and *B. abortus* strain RB51 for cattle, *B. melitensis* strain Rev 1 for sheep and goats, and *B. suis* 2 for swine. Although the Rev 1 vaccine is highly infectious for humans, it is considered to be the best vaccine for the control of brucellosis in sheep and goats [22]. Currently, antibiotic prophylaxis would be the only option to prevent infection following the deliberate released brucellosis. There is no experience with antibiotic prophylaxis in cases exposed to *Brucella* bacteria. The current recommendations are based on the derived data from accidental laboratory exposure. Table 6.4 summarizes the current recommended antibiotic prophylaxis [1, 4, 8, 9, 72].

In conclusion, *Brucella* spp. is highly infectious via the aerosol route making them an attractive pathogen for those with nefarious intentions. The global biologic risk of biological attack is increasing for a variety of reasons. Scientists need to focus their efforts on the development of a new safe and effective human *Brucella* vaccine and new drugs for the treatment of *Brucella* infection. When preparing biodefense systems, countries should consider countermeasures against *Brucella* spp. along with other priority biological agents.

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Burkholderia mallei and Glanders

7

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7.1 History of Glanders

7.1.1 History of *Burkholderia mallei* and Glanders

Glanders is an obligate mammalian infectious disease caused by the Gram-negative bacterium *Burkholderia mallei* [1–4]. Mainly affecting equids (horses, mules, and donkeys), glanders can also be contracted by humans through inhalation or percutaneous inoculation [2–5]. *B. mallei* is a member of the complex phylogeny of *Burkholderia* genus including more than 60 different species thriving in a wide range of environmental niches [6–9]. Among the members of this genus, plant pathogens and other saprophytic bacteria exist, as well as human pathogens such as *B. pseudomallei*, the causative agent of melioidosis, *B. thailandensis*, as well as members of the *B. cepacia* complex, which are important pathogens in cystic fibrosis patients [6–8, 10, 11]. The inability of *B. mallei* to survive for extended periods of time outside a host is thought to be the result of reductive evolution from its close relative *B. pseudomallei*, which can survive for long periods in the environment [2–4, 12].

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Glanders, or sometimes referred to as farcy when the disease manifests in the cutaneous form, is among the oldest disease recorded [4]. The initial description of the clinical signs of glanders dates back to 425 B.C.E. by the Greek physician, Hippocrates [2, 4]. This description dates to the thought of a disease to be the result of an imbalance of the humors in the body. This concept explained that infections arose from an imbalance in four vital fluids and remained the central thought of western medicine until the late 1860s [13]. The glanders treatment as initially described was the application of wine and olive oil into the nostrils [13]. Almost 100 years after its initial description, Aristotle grouped glanders under the general description of diseases to infect both animals and humans (zoonotic diseases) and gave it the name 'melis' in Greek meaning "severe disease" (or mallus in Latin meaning, "malignant disease") [3, 13]. The first documented recognition of glanders as contagious disease was by the ancient Roman historian Vegetius in the fifth century C.E., who recommended the separation of infected horses to prevent the spread of the disease [14]. The debate whether glanders was contagious remained deeply divided until Viborg demonstrated transmissibility in 1797 [3]. Later, in 1876, the disease came to be accepted as contagious after the pioneering work by Pierre Francois Olive Rayer, who inoculated a horse with pus from a groom that died of glanders—and the animal developed the disease [13].

The field of modern microbiology radically shifted in the late nineteenth century when Robert Koch outlined his postulates as guidelines to describe microbial disease in humans. It was this revolution that allowed Frederich Loeffler to isolate the glanders organism from the lung and spleen of an infected horse, thereby ending the debate on the etiological agent of glanders [2, 5]. The first extensive clinical study of glanders was conducted during the U.S. Civil War by Drs. John R. Page and John J. Terrell; both of whom were U.S. Confederate army surgeons [2, 13]. Since its initial isolation, *B. mallei* has been re-classified as a member of different genera, including *Bacillus*, *Corynebacterium*, *Mycobacterium*, *Loefflerell*a, *Pfeifferella*, *Malleomyces*, *Actinobacillus*, and *Pseudomonas* [3–5]. Most recently, in 1992, *B. mallei* was classified in the genus *Burkholderia* on the basis of 16S ribosomal DNA sequences, DNA-DNA homology, and physiological characteristics, such as cellular lipid and fatty acid composition, as well as phenotypic characteristics [15]. Extensive control programs by many countries including US, Canada, and the UK, in the latter part of the twentieth century led by increasing information about the pathology, epidemiology, and diagnostics allowed the eradication of the disease in these countries [2, 4]. Today, there are few reported cases of glanders in humans; the last of human infection in the USA was recorded in 2000. Nonetheless, glanders continues to occur in parts of Asia, South America, Northern Africa, and it is endemic in Iraq, Pakistan, India, Mongolia, and parts of Brazil [3, 16]. Due to the number of recent outbreaks in the last 10–20 years (Fig. 7.1), glanders has retained its classification as a re-emerging disease [3, 4, 17, 18].

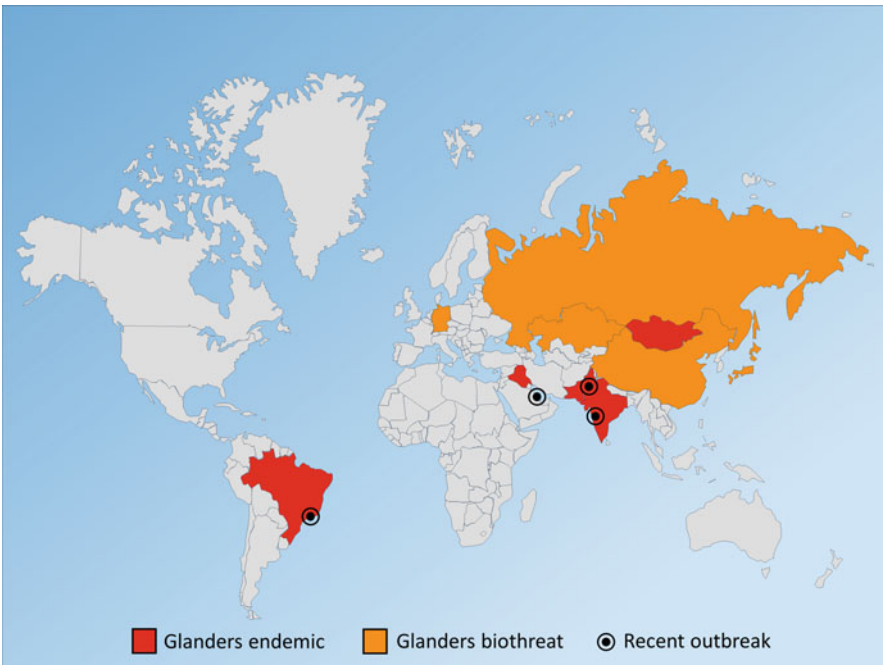

Fig. 7.1 World map of glanders endemicity, historical bioweapon use, and recent outbreaks up to 2016. Previously associated countries the glanders as a biothreat, either by direct use or direct attack (orange). Countries with endemic cases (red). Recent outbreaks up to 2016 (black circle)

7.1.2 *B. mallei* as a Bioweapon: A Historical Perspective

The historical use of *B. mallei* use as a bioweapon is extensive and dates back to the fourth century during the reign of Constantine the Great [3]. Given that glanders predominantly affects horses, this disease carried a significant economic burden on what was considered to be the primary source of transportation [13]. Glanders affected the Crusaders causing infections to their horses [3, 13]. The disease was considered dangerous enough by Louis XV that he founded the first veterinary school at Lyons, with the purpose of studying the effects of glanders on the French cavalry [13]. The first record of glanders in the USA was during the American Revolution among the British cavalry, though it was not of any significance until the Second Seminole War from 1835 to 1842 [13]. Though no record of intentional use, the U.S. Bureau of Animal Industry admitted in 1890 to have introduced glanders in Mexico during the Mexican-American War through diseased horses of the U.S. Army. Its continued use in warfare was extensively documented during the American Civil War, where the Confederate army had left several infected horses behind after battles causing a significant number of infections in horses, mules, and civilians alike [13]. However, the most impressive numbers of glanders-related fatalities came from the mishandling and selling of infected horses for the Federal

Army in the Giesboro depot in 1864. This base was the Federal Army's largest horse supplier. This incident was the first large-scale infection of animals where, despite incomplete epidemiological records, estimates suggest almost a quarter of a million animals became exposed to the disease and a record 188 dead animals in a single day [13]. After the end of the Civil War, many of the animals left in these depots, as well as the soldier's animals were either distributed or sold to the public and few records remain to their outcome [13].

The warfare-associated cases of *B. mallei* resurged up until the beginning of the twentieth century during World War I (WWI), in which over 58,000 horses that belonged to the French army became infected with *B. mallei*, mainly by transmission from captured Russian horses [4, 8, 19, 20]. WWI also marked the first intentional release of *B. mallei* as a bioweapon by German against allied forces; Germany sent agents carrying microbial cultures to several allied countries shipments of allied horses, mules, cattle, and sheep [19]. The use of *B. mallei* as a bioweapon continued in WWII by Japanese forces when the bacterium was used against horses, civilians, and war prisoners in the war base of Píngfáng, located in the Japanese puppet state of Manchukuo, China [4, 21]. Reports of *B. mallei* as a bioweapon emerged up to the latter portion of the twentieth century with claims that the Soviet Union utilized the bacterium against Mujaheddin horses during the Afghan war from 1982–1984 [4, 12]. After this report, no further evidence has been documented about the use of *B. mallei* as a bioweapon. However, given its susceptibility to human infection, lack of effective therapeutics, resistance to treatment, and its potential use as a bioweapon, *B. mallei* is classified, since 2000, as a Tier 1 Select Agent by the US Department of Health and Human Services and the Centers for Disease Control and Prevention [2, 20].

7.2 Pathogenicity Mechanisms

7.2.1 Adhesion and Invasion

The genome of *B. mallei* (5.8 Mb) contains two circular chromosomes, chromosome 1 (3.51 Mb) and chromosome 2 (2.32 Mb) [22, 23]. The majority of the genes in *B. mallei* share ~99% DNA sequence identity with *B. pseudomallei*; however, more than 1000 annotated genes are absent in *B. mallei* [23].

B. mallei is a facultative intracellular pathogen that can adhere, invade, and multiply inside phagocytic and epithelial cells. In a study by Memisevic et al., *B. mallei* proteins that were associated with host-pathogen interactions were aligned, and the results showed its genome contains PilA and VgrG protein-encoded BMA0278 and BMA0446 genes, respectively [24]. The alignment predicted roles of these two proteins to be associated with cell adhesion and promotion of bacterial survival [24]. PilA is a type IV pilin characterized as having a role in the virulence of *B. pseudomallei* infection. Loss of PilA resulted in reduced bacterial virulence in both a nematode and mouse models of infection, suggesting a role in adherence to

epithelial cells [25]. The role of the *B. mallei* VgrG protein was predicted to have a role in cell adhesion, promotion of bacterial survival, and replication [24].

An in vitro study using human respiratory epithelial cell line A549 and mouse respiratory epithelial cell line LA-4 demonstrated that *B. mallei* poorly adhered and failed to invade both cell lines [26]. *B. mallei* was phagocytosed by murine alveolar macrophage cell line MH-S and intracellular survival decreased [26]. The *B. mallei* adhesion efficiency was also evaluated in apical surface binding of human alveolar type II cells (ATII), *B. mallei* was shown to adhere to ATII cells but significantly less than *B. pseudomallei*. Likewise, *B. mallei* was slightly phagocytosed by human monocytes-derived macrophages (hMDM) and it poorly invaded and replicated in ATII cell lines [27]. The protein product of the *B. mallei boaA* gene showed significantly increase adherence to human epithelial cell lines, (HEp2), A549, and normal human bronchial epithelium (NHBE) when expressed in *Escherichia coli.* In contrast, the adherence was reduced about 50% in a *B. mallei* ATCC 23344 *boaA* mutant strain [28].

7.2.2 Secretion

7.2.2.1 Type III Secretion System (T3SS)

The T3SS locus of *B. mallei* ATCC 23344 was analyzed by using genetic alignment to the *B. pseudomallei* T3SS locus. The mutagenesis of T3SS of *B. mallei* ATCC 23344 drastically reduced virulence in a BALB/c mice model [29]. Phenotypes of known *B. mallei* T3SS virulence proteins have been reported in an animal model of infection, namely, BMAA1521 (BopA), BMAA1528 (BipD), BMAA1530 (BipC), BMAA1531 (BipB), BMAA1523 (BopE), BMAA1538 (BsaU), BMAA1525 (BapB) and BMAA1865 [29]. BopA is an effector protein required for bacterial internalization and promoting bacterial survival [26, 30]. BALB/c mice given *B. mallei* Δ*bopA* had increased survival, as compared to wild-type *B. mallei* infection [26].

The BipD protein is involved in transcription regulation and formation of the secretion needle top protein. BipD is associated with virulence in *B. pseudomallei* by facilitating the bacterium's ability to invade non-phagocytic cells. BipD mutant was found significantly more attenuated in both BALB/c and C57BL/6 mice [31]. The secreted effector and translocator protein BipB, has a major role in multinucleated giant cell formation in *B. pseudomallei*. The formation of multinucleated giant cells was reduced in a *B. pseudomallei* lacking the BipB effector protein. Furthermore, *B. mallei* BipB was predicted to have a role in bacterial internalization [32].

The loss of BopE did not show attenuation during *B. pseudomallei* infection in BALB/c mice [30, 31]. BsaU and BapB proteins have an important role in bacterial escape from endocytic vesicles [33], and interference with host ubiquitination [24], respectively.

Furthermore, the putative role of BMAA0429 (cytidylate kinase, Cmk), BMA2469 (transkelolase, Tkt), BMA3281 (flagella M ring protein, FliF) and BMAA1619, were predicted using host-pathogen interaction alignments. Cmk is

predicted to be a regulator of T3SS secretion, whereas Tkt and FliF were proposed to have a function in virulence during bacterial internalization, interference with host cytoskeleton, and promotion of bacterial survival [24].

7.2.2.2 Type VI Secretion System (T6SS)

B. mallei ATCC 23344 T6SS gene cluster 1 (T6SS-1) was characterized and described as an essential virulence factor in the pathogenesis of glanders. This cluster contains T6SS-associated genes (BMAA0744-0730) known as *tssA*-*tssN* loci, as well as hemolysin co-regulated protein 1 (*hcp1*), *clpV1*, *vgrG1* (encoding a valine glycine repeat protein 1) and *icmF1* [34]. All T6SS genes are required for full virulence of *B. mallei* in a hamster model of infection. In addition, the T6SS is regulated by the VirAG two-component system [34]. The VirAG-related genes encompass *bimA*, *tssA*, *hcp1* and *tssM*, and it regulates the expression of T6SS-1 in minimal media (M9G), as well as M9G plus casamino acids [35]. The Hcp and VgrG proteins are the main components in T6SS apparatus. In addition, the structures of Hcp and VgrG are homologous to phage tail tube and T4-phage baseplate proteins, respectively [36]. Hcp1 plays a significant role in multinucleated giant cell formation in murine macrophages during *B. pseudomallei* infection [37]. Likewise, this protein serves as a structural component and a secreted protein that play important role in *B. mallei* pathogenesis [34, 38].

The *tssN* was analyzed by bioinformatics and it was proposed to play a role in the interference with host signaling and ubiquitination [24]. Subsequently in an in vivo model, the function of *tssN* was evaluated and its mutant showed decreased intracellular survival and reduction or delay in the formation of multinucleated giant cells [39]. Moreover, 67% of mice that were given aerosolized *tssN* mutant strain showed survival up to 21 days after exposure to wild-type *B. mallei* [39]. The T6SS-1 neighboring gene *tssM,* was shown to have no effect on intracellular survival or multinucleated giant cell formation in *B. mallei*-infected murine macrophages [40, 41].

7.2.3 Quorum Sensing (QS)

The QS systems in *B. mallei* are known as BmaI/R and are comprised of two *luxI* (*bmaI1* and *bmaI3*) and four *luxR* (*bmaR1* and *bmaR3*) genome homologs [42]. The *bmaI1* and *bmaI3* produce *N*-octanoyl-*L*-homoserine lactone (C8-HSL), while *bmaR1* and *bmaR3* respond to signals produced by *luxI* homolog [43]. The QS system genes of *B. mallei* are in chromosome 2. First, the BmaI1/R1 are present in all *B. mallei* strains and share similarity to BpsI1/R1 in *B. pseudomallei* [43]. BmaI3 produces *N*-3-hydroxy-hexanoyl-HSL ($3OHC_6$-HSL), *N*-3-hydroxy-octanoyl-HSL ($3OHC_8$-HSL) and *N*-3-hydroxy-decanoyl-HSL ($3OHC_{10}$-HSL). The BmaR3 responds to $3OHC_8$-HSL which is the most abundant compound produced when expressed as recombinant in *E. coli* [42]. The other QS system, LuxR homologs BmaR4 and BmaR4 have are not completely characterized.

7.2.4 Autotransporters

The autotransporter family is a diverse and largely virulent group of proteins that influence Gram-negative bacterial pathogenesis [44]. *B. mallei* ATCC 23344 genome has two homologs (BMA1647 and BMAA1263) of *B. pseudomallei* classical autotransporters and six (BMA1027, BMA0840, BMAA0649, BMAA1324, BMAA0810 and BMAA0749) trimeric autotransporter adhesins. The classical autotransporters BMA1647 and BMAA1263 are predicted to have the biological characteristic as putative lipase/esterase and serine proteases [45], while all six trimeric autotransporter adhesins were shown to have immunogenic properties with glanders sera [45]. The *B. mallei* ATCC 23344 locus BMAA0649 was annotated to be *boaA,* a gene that was shown to have an important role in host cell adhesion process [28]. The C-terminal sequence of BMAA0810 protein contains a *Yersinia* adhesion A (YadA) domain, which is suggested to be a cell surface binding protein that modulates host-cell interactions [45].

The BimA homolog of *B. mallei* ATCC 23344 has the ability to stimulate actin assembly and restore tail formation during infection of murine macrophage-like J774.2 cell line [46]. Interestingly, the *B. mallei bimA* gene product was reported to be non-immunogenic in a test with *B. mallei*-infected horse sera [45], and not required for virulence in a Syrian hamster model of acute glanders [34].

7.3 Epidemiology

7.3.1 Hosts

Glanders is a zoonotic infection caused by *B. mallei*, a pathogen that needs an animal host to survive. The primary natural reservoir for *B. mallei* are members of the family Equidae (horses, mules, donkeys), from which acute forms of the infection occur most frequently in donkeys and mules, with high fever and respiratory signs, whereas horses generally present a more chronic course and they may survive for several years, especially in endemic areas [4]. Interesting, the name glanders originated from the lymphangitis and lymphadenopathy (glands) that are associated with disease in horses. In the case of cutaneous manifestations, the disease is called Farcy. Although less susceptible, humans, and occasionally felids, camels, bears, wolves and dogs are susceptible to infections. Other carnivores may become infected by eating infected meat; however, cattle and pigs are resistant [4].

7.3.2 Sources of Infection and Transmission

Transmission of *B. mallei* from horses or other solipeds to humans appears to be uncommon, even when frequent and close contact with infected animals is occurring [47]. Despite low incidence of animal to human transmission, occupational exposure of the animal handlers remains a key risk factor, particularly veterinarians, soldiers,

slaughterhouse personnel, farmers, and other horse handling professions. Human-to-human transmission is also rare. However, it may occur during occupational exposure in medical practice or at autopsies [47]. In the case of laboratory workers, they have rarely been infected; however, close contact with high concentrations of virulent bacteria might put them at high risk for infection. In the case of animal-to-animal transmission, the most common source of infection appears to be ingestion of contaminated food or water likely via discharges from the respiratory tract or ulcerated skin lesions from carrier animals [47]. Animal density and proximity favor spread as well as stress-related host factors.

7.3.3 Occurrence

Early in the twentieth century, glanders disease was still widely present worldwide; however, the effective use of veterinary interventions (large-scale culling) and national control programs initiated between the 1940s and 1950s had significantly reduced the prevalence of this disease. Irrespectively of the implementation of these interventions, glanders continues to be reported in Brazil, India, Iran, Iraq, Pakistan, Turkey, and the United Arab Emirates and it is thought to be endemic in various areas of Asia, Africa and South America [3, 5]. In such countries, economic and cultural circumstances may hinder culling of asymptomatic animals, enabling the persistence of glanders disease.

In recent years, several outbreaks occurred in horse populations in Asia, including Western Asia (Afghanistan, Kuwait, Iran, Iraq, Pakistan, Syria), Africa, and South America (Brazil). Further, recent rise of glanders cases in horses, in combination with worldwide horse trading, results in the potential for the disease to be re-establish in countries in which it has been previously eradicated (glanders is now considered a re-emerging disease) [3], and posing new risks for human infections.

7.3.3.1 Glanders in Bahrain

In April 2010, a large outbreak of glanders was reported in Bahrain, an archipelago of 36 islands in the Persian Gulf off the eastern coast of Saudi Arabia, and home to about 6500 horses. Bahrain was considered a glanders-free country until horses imported from Syria via Kuwait were suspected of introducing glanders [48]. By September 2010, the outbreak was considered resolved. However, in January 2011 the disease reoccurred in the same region of the country and, at the end of the investigation, 50 horses and one camel tested positive and the bacteria was isolated from 8 horses and the camel.

Genotypic and comparative analysis from the bacterial strains isolated in 2010 to those of a prior *B. mallei* outbreak in the United Arab Emirates [UAE] in 2004, indicated that the samples from the outbreak in Bahrain were separated into two distinct clusters, suggesting that two independent but simultaneous strain introductions took placed and caused the outbreak [48]. To further confirm, multilocus variable number tandem repeat analysis of the *B. mallei* strain isolated

from a diseased camel in Bahrain revealed close genetic proximity to UAE strain Dubai 7, confirming that glanders disease in this animal was the result of the outbreak but caused by a second strain [16].

7.3.3.2 Gladers in Brazil

Brazil is considered a glanders endemic country and historically, foci of glanders occurred with more frequency in the north and northeast of the country; for example, in equids of the "Zona da Mata" in the states of Pernambuco and Alagoas [49]. In this country, at least 18 states have foci of glanders, which result in notification of glanders disease incidence to the World Organization for Animal Health (OIE; http://www.oie.int/en/animal-health-in-the-world/animal-diseases/) and this organization recommends euthanization of the infected animals.

The prevalence of glanders disease in Brazil was in the spotlight recently during the 2016 Olympic Games at Rio (Fig. 7.1) [50]. At the end of July 2015, it was confirmed that at least 17 horses were diagnosed with glanders, and all the animals were quarantined or euthanized in Cananea Island, near Sao Paulo. This information gained relevance prior to the start of the Olympic equestrian events because it was believed that some of the horses at Cananea Island came from the Deodoro military complex, a place housing the Army Equitation School, and which is located very close (0.35 mi) to the Olympic Equestrian Centre (COH). The ministry of Health confirmed that despite the threat of glanders near Rio de Janeiro, the situation was not a threat to health security of Olympic events; however, the COH was placed in sanitary isolation since February of 2015. Because of these actions, all the equestrian events continued without an incident [51].

7.3.3.3 Glanders in India

In this country, glanders was detected among horses, donkey and mules, but this disease has been restricted to certain geographical pockets with sporadic cases detected in the 80's–90's. Because the animals are used for transport, there is a constant, perceptible threat for the re-emergence of this disease in equines, mainly due to work stress and cross border exposure. Considering that historically the disease in India was restricted to certain pockets with sporadic cases, it is important to detect and report any new cases in the field. Such monitoring activities resulted in the identification of outbreaks occurring in different Indian States from 2006 to 2010 (Fig. 7.1) [52] and culminating with a major glanders outbreak among equines in the state of Maharashtra [18]. During these outbreaks, a total of 164 equids were found positive and following the provision of Prevention and Control of Infectious and Contagious Disease in Animals Act 2009, the infected animals were euthanized and control measures were implemented [52, 53].

More recently, the potential for human exposure to the disease have been of significant concern due to the detection of glanders among animals carrying pilgrims to the Holy Cave Shrine of Mata Vaishno Devi Ji back and forth from Ban Ganga to Bhawan, raising concerns over safety of the pilgrims as well as people living in holy town of Katra, the base camp where the shrine is located (Fig. 7.1) [54]. During pilgrimage, large numbers of working animals are used (estimates indicated approx.

5000 mules) to carry the pilgrims, which indicates that one single case can quickly spread to other animals and humans. Overall, from 1704 blood samples taken from animals, 17 tested positive for the disease and all the animals were euthanized [54]. This outbreak is an example of the need of implementing control actions to prevent dissemination because in this case, the high number of mules generating massive amounts of mule dung along the 13 km track to Mata Vaishno Devi shrine can posed a significant threat to residents and visitors as they have been consuming and using contaminated water supplies.

7.4 Treatment and Vaccines

7.4.1 Antibiotic Treatment and Novel Therapeutic Approaches

The understanding of *B. mallei* treatment is not as extensive as for its counterpart *B. pseudomallei* [1, 55–57]. This limitation is due to poor understanding of *B. mallei* pathogenesis [1, 55, 57]. Therefore, treatment for glanders is like melioidosis, and development of new approaches is often based on our knowledge of *B. pseudomallei* pathogenesis [57]. The antibiotic susceptibility and resistance of *B. mallei* are often treated equally to *B. pseudomallei* in clinical cases [57]. Assuming rapid and accurate diagnosis of *B. mallei*, treatment for human cases of glanders included mixed antibiotic therapy [55–57]. This considerable gap in our understanding for clinical treatment is due in part to the small number of reported cases [57].

Treatment of glanders involves intravenous administration of imipenem, ceftazadine, and doxycycline for 2 weeks, followed by and eradication phase using an oral administration of azithromycin and doxycycline for an additional for 6 months [2, 4, 57]. This treatment regimen is mildly effective and is associated with relapse of the disease [2, 58]. The increasing challenge of antibiotic-resistant variants of *B. mallei* often require supportive therapy including abscess drainage to alleviate localized infection [2, 57]. Unlike *B. pseudomallei*, *B. mallei* is susceptible to imipenem, ceftazidime, ciprofloxacin, and piperacillin, as well as aminoglycosides, because of the absence of the ArmAB-OprA antibiotic resistance pump [2, 57]. Novel anti-glanders drugs focus on mitigating the challenges of prolonged treatment times, route, and severity of the infection. Granulysin is a broad-spectrum antimicrobial peptide member of the saponin-like family of proteins previously evaluated against *B. mallei* [57, 59, 60]. Granulysin delivery may serve as a therapeutic drug against *B. mallei*, given its activity against bacterial cell viability [57, 59, 60]. Another novel therapeutic proposed against *B. mallei* are silver carbine compounds [57]. The antimicrobial activity of silver against Gram-positive and negative bacteria is widely studied and established [57, 61–63]. Two silver carbene compounds have antimicrobial properties against *B. mallei* with more susceptibility to these complexes than *B. pseudomallei* [57]. However, the systemic administration of metal compounds is still not approved for clinical use. In conclusion, the limited knowledge of both the pathogenesis and drug susceptibility of *B. mallei* has impeded the development of novel drugs and therapeutics.

7.4.2 Vaccines

Currently, there is no licensed vaccine for either human or animal use against glanders [1, 56, 64]. However, the different array of vaccine platforms against *B. mallei* can protect mice against acute disease [56, 64]. Nonetheless, most attempts to protect against infection fail to provide complete protection against chronic disease across a broad range of exposure routes, other than at very low exposure doses [56, 64]. In many of the approaches for vaccination against *B. mallei*, protection often dependent on immunization by the same route as infection [1, 55, 56].

7.4.3 Whole-Cell Vaccines

7.4.3.1 Live-Attenuated Vaccines

Live vaccines against glanders present the most efficacious candidates to date providing rapid, broad, and long-lasting protection, in the absence of adjuvants [1]. However, the use of live attenuated vaccine platform for humans raises safety concerns with the potential for pathogen reversion and possible adverse effects, especially in immunocompromised individuals [1, 17, 64, 65]. Nonetheless, some concerns using this vaccine approach may be ameliorated if strains are engineered to include multiple mutations, deletions, or mechanisms to prevent reversion and limit the potential for host persistence [1, 64]. Several mutagenesis approaches to create live-attenuated vaccines have included the *tonB* iron transport system [66], endoproteases (*cptA*) [67], quorum sensing (*bmal3*) [68], and amino acid biosynthesis (*ilvl*) [69]. These mutant strains were previously tested in murine inhalational, intranasal, or systemic glanders models, ranging in their attenuation and protective efficacy [29, 66, 67]. *B. mallei ilvl* (amino acid synthesis) deletion strain provided short-term resistance to high and low *B. mallei* aerosol exposure doses, with 25–50% mice surviving for 1 month post-infection [69]. Mice vaccinated with *B. mallei* Δ*bmal3* mutant (quorum sensing) exhibited 30% survival at 11 days post-infection (dpi) when exposed to wild-type *B. mallei* using an aerosol model of infection [29]. *B. mallei ctpA* (endoprotease) mutant strain showed partial protection in an i.p. model of glanders with 75% survival in mice after 15 dpi [67]. One of the most promising vaccine candidates is the *B. mallei* strain CLH001 [17]. This strain includes a deletion in both *tonB* (iron transport) and hemolysin coregulated protein 1 (*hcp1*) [17]. Vaccination with CLH001 resulted in complete protection up to 21 days with no liver or lung colonization [17]. However, some bacteria were recovered from the spleen at a higher exposure dose. Taken together, many of these attenuated vaccines showed partial to full protection against acute aerosol through different exposure routes but fail to provide full protection against chronic disease [17]. Interestingly, the best protection against lethal intranasal glanders and melioidosis infection is using the live attenuated *B. mallei tonB* mutant [64, 66]. Although this single mutation can provide protection against both pathogens, further safety and efficacy studies are required.

7.4.3.2 Killed Vaccines

Killed vaccines are an alternate approach to live-attenuated vaccines but they are often unable to induce cell-mediated immunity, presenting a disadvantage in creating sterilizing immunity [1, 56]. The use of adjuvants may provide effective immune responses while maintaining their safety [56]. Amemiya et al. demonstrated that vaccination with non-viable *B. mallei* induced a mixed Th1- and Th2-immune responses in mice [70]. Mice vaccinated with heat-killed *B. mallei* via i.p. route showed a mean survival time of 40% against a lethal exposure of ~20 LD_{50} [71]. Heat-irradiated, irradiation-inactivated, as well as irradiated capsule-mutant inactivation platforms were administered subcutaneously (s.c.) to mice [71]. These different killed vaccine platforms resulted in protection ranging from 80–100% at 21 dpi at low dose [1], but failed to protect against high exposure doses [1]. However, incorporating IL-12 into the s.c.-administered irradiated vaccine increased protection by up to 60% [72]. Furthermore, the incorporation and co-delivery of IL-12 as an adjuvant as well as activator of IFNγ-producing T-cells and Th1 responses may be necessary to enhance humoral and cellular immune responses to killed *B. mallei* [72]. A formalin-inactivated vaccine was developed in Russia using wild-type *B. mallei* (strain 11) [65]. This vaccine protected 70% of animals when adjuvanted with aluminum hydroxide in Guinea pigs against *B. mallei* [65]. A single s.c. injection of this vaccine strain delivered at 4×10^9 CFU is designed to protect against local cutaneous and pulmonary glanders and elicits elevated antibodies in sera up to 1-year post-vaccination in 27.3% of humans [65].

7.4.4 Subunit Vaccines

Subunit vaccines have historically been used as a safe alternative to whole-cell vaccination, but with varying levels of efficacy [1, 2, 56]. Subunit vaccination induces a Th2-biased immune response to single proteins or unconjugated LPS [1]. This response is thought to be ineffective against bacteria like *B. mallei* capable of replicating intracellularly [1, 56]. This reaction can potentially be more efficient in polyvalent vaccines that combine multiple antigens and are capable of generating protection against heterologous strains and different routes of infection [1, 73, 74].

7.4.4.1 Proteins Subunit

Subunit-based vaccines against *B. mallei* have mainly developed using a single protein from the best-known virulence factors [1]. Vaccination of mice with individual or combination of *B. mallei* proteins Hcp1, BimA, BopA, or *B. pseudomallei* LolC resulted in survival rates ranging from 75–100% [75]. The most significant efficacy was seen in mice immunized with BopA and BimA which led to bacterial clearance from the lungs but not in the spleen, and 100% survival in mice at 21 dpi exposed to 2 LD_{50} of wild-type *B. mallei* [75]. The best protein subunit candidate to cross-protect against intranasal glanders, and melioidosis to date remains BopA [75].

7.4.4.2 Synthetic Vaccines

Among the most notable synthetic vaccine is the gold nanoparticle (AuNP) glycoconjugate which was used as a platform for delivery of protein subunits conjugated to *B. thailandensis* LPS [76]. The flagellin protein, FliC, were previously tested using the AuNP delivery system against aerosol exposure in a murine and rhesus monkey model of infection [76]. Mice challenged with wild-type *B. mallei* were nearly 80–100% protected against intranasal exposure with ~3.5 LD_{50} of *B. mallei* up to 21 days [77]. When tested in rhesus monkeys against ~1 × 10^4 CFU *B. mallei* exposure, increased protection was observed in sub-cutaneous-immunized animals [76]. Another safe alternative for antigen delivery is DNA vaccination using a plasmid-encoded bacterial protein expressed in eukaryotic cells. However, results have shown varying efficacy in animal studies as a result of low expression of antigens [1, 64]. To further identify protective antigens against *B. mallei*, expression library genetic immunization was used by delivering *B. mallei* ORFs before exposure to ~2 LD_{50} of *B. mallei* [78]. Several ORFs conferred a significant difference in the extended time of death with 87.5% survival in exposed mice at 20 dpi. From the characterization of these protective ORFs, 12 novel vaccine candidates were identified [78].

7.5 Diagnostics

Diagnosis of glanders based on clinical features alone are not available and require a positive culture of *B. mallei* from clinical samples such as blood, exudate, or pus from abscesses [79]. Culture methods are customary used and often serve as the gold standard for diagnosis. However, it is recommended that a culture of an isolate be incubated for 72 h at 37 °C due to slow growth of the bacteria, followed by confirmation with biochemical tests or PCR confirmation [80]. Alternative serological tests and molecular techniques were developed to increase sensitivity and specificity for the diagnosis of glanders.

7.5.1 Serology Tests

7.5.1.1 Agglutination Test

Latex agglutination assays are currently used to identify *B. pseudomallei* and related bacteria in endemic areas, such as South-Eastern Asia and northern Australia. This test is based on monoclonal antibodies (MAbs) to a surface-exposed exopolysaccharide [81–84]. Duval et al., developed the antibody-latex suspension based on the 4B11 MAb that is specific to *B. pseudomallei* exopolysaccharide. Using single colonies, 33 *B. mallei* isolated from China, USA, India, Turkey, Burma, Hungary, England and Iran were confirmed positive (100% sensitivity) in this test [85].

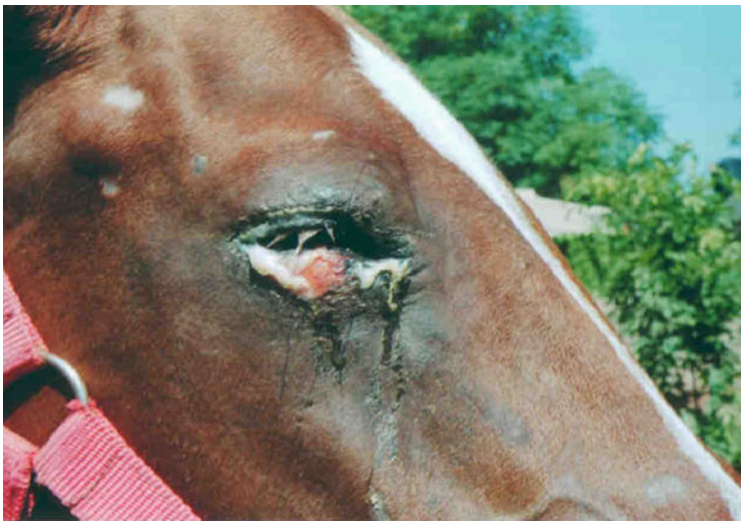

Fig. 7.2 Severe mallein reaction in geldings with glanders (Picture courtesy of an unidentified Pakistani Veterinarian)

7.5.1.2 Complement Fixation Test (CFT) and Malleinization

The CFT test has been used to detect glanders in equines and later recommended by the World Organization for Animal Health (OIE) for serological test in international animal trading. The specificity and sensitivity of CFT are significantly affected by the quality of the *B. mallei* antigen used [86], and incubation temperature [87]. Three commercially available CFT antigens from the c.c.pro (c.c.pro GmH, Oberdorla, Germany), Central Veterinary Institute of Wageningen UR (CIDC), and the United States Department of Agriculture (USDA) were compared by using 410 serum samples from animals. The evaluation results by immunoblot assay found highest sensitivity (97.5%) in the CIDC antigen, followed by c.c.pro (96.5%), and contrasting with the USDA antigen which showed reduced sensitivity (61.19%), but 100% specificity [88]. However, both CIDC and c.c.pro antigens showed low specific detection in sera from endemic animals. As a result, the combination of CFT and immunoblot has been highly recommended for the serodiagnosis of glanders [89]. The mallein allergic test is frequently used in conjunction with CFT for glanders identification in infected animals (Fig. 7.2). The mallein purified protein derivative is extracted from *B. mallei* cultures and used as antigen to induce a cellular immune response in animals [86, 90]. The CTF is still used to screen glanders in animal in the United States, while the malleinization test is used to confirm positive animals from CFT [91].

7.5.1.3 Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA diagnostic for glanders was developed because the malleinization test showed limited sensitivity in clinically advanced cases and reported false positive/

negative results [86]. Recombinant proteins specific to *B. mallei* were used to detect antibody by indirect ELISA. Unique DNA sequence at 5′ end of BimA, provided a specific recombinant antigen and has been used for detecting the anti-glanders antibodies with indirect ELISA. The results showed 100% sensitivity and 98.88% specificity. In addition, BimA did not react with serum samples from melioidosis or healthy individuals [92]. Moreover, two complete (0375H and A0350H), and two truncated (0376TH and 0375TH) proteins purified from *B. mallei* NCTC 10230 were evaluated for their antigenicity and ability to detect *B. mallei*-specific antibodies in equine sera by indirect ELISA. Recombinant protein 0375TH and 035TH exhibited 100% sensitivity and specificity for diagnosis of glanders. Importantly, these proteins did not cross-react with sera from melioidosis patients [93]. Likewise, no cross-reactivity results were reported from non-melioidosis patient serum samples when using the recombinant truncated TssB protein from *B. mallei* as a coated protein. This indirect ELISA diagnostic showed a 99.7% specificity and 100% sensitivity [94].

7.5.2 PCR Based Tests

Prompt diagnosis of the disease continues to be a limitation to increase patient survival in case of infection. The PCR methods was developed to detect low bacterial number in various clinical specimens due to the sensitivity, specificity, and accuracy associated with the technique [95].

Specific single and multiple target genes were selected to permit sensitivity and differentiation of *B. mallei* based on specific DNA sequences. The 16s rRNA genes from 56 *B. pseudomallei* and 23 *B. mallei* genomes from different geographical regions and years and origin of isolates, were cloned and sequenced using specific primers. Twenty-two of 23 *B. mallei* isolates showed sequence identity. This work indicated that 16s rRNA gene sequence used with appropriate primers can identify and differentiate *B. mallei* from *B. pseudomallei* and can be faster diagnostic compared to biochemical test and traditional colony morphology observations [96]. The target of *B. mallei* 16s rRNA gene was used to confirm the infection with *B. mallei* in dromedaries in Bahrain in 2004 [16].

As a non-motile bacterium, the *B. mallei* flagellin (*fliC)* gene sequence was identified and shown to contain a truncation at position 798 from G to C in comparison with *B. pseudomallei* [97]. The *fliC* gene was used along with TaqMan probes targeting with 16S rDNA, but it could not achieve the discrimination between *B. mallei* and *B. pseudomallei* [98–100].

The rapid 5′-nuclease real-time PCR assay was developed for detection of *B. mallei* DNA. The primers were designed based on a known flagellin P (*fliP*) sequence from *B. mallei* ATCC 23344 and *B. pseudomallei* K96243. These primers were specific to the *fliP*-IS 407A region of *B. mallei*. With this method, all 19 *B. mallei* strains isolates were amplified (100% sensitivity). Application of *fliP* targeting gene by PCR assay was used to detect *B. mallei* DNA fragments in pure cultures and clinical samples from equine glanders in UAE. All 20 of *B. mallei*

strains isolated from different environmental or clinical specimens in this country were able to be amplified (100% sensitivity), whereas other closely related burkholderiae resulted in a negative result (100% specificity) [101].

The PCR studies targeting the *B. mallei* intracellular motility A gene (*bimA*) were developed based on the unique DNA sequence within the 5′ region of the *B. mallei* $bimA_{ma}$ [46]. PCR primers designed to $BimA_{ma}$ showed specificity to 29 of 31 *B. mallei* isolates (94%) when compared to other unique nucleotide sequences that are highly conserved among all virulent *B. mallei* isolates [102]. Furthermore, two different primer pairs were designed and they could detect the *B. mallei bimA* gene by real-time PCR with 100% accuracy. These two assays also detected *B. mallei* in lungs, spleen and livers of infected BALB/C mice while it was not detected in blood samples [102].

The *B. mallei* and *B. pseudomallei* genomes encode approximately 40–50 copies and 5 copies of transposase *ISBma2*, respectively. This transposase sequence was used as signature sequence for primer design incorporated with *B. mallei* signature sequence *mau*, which is a member of the phage integrase protein family. The signature sequence was amplified by qPCR using purified DNA from *B. mallei* strains. The assay presented high sensitivity for *B. mallei* and *B. pseudomallei*. This multiplex qPCR method was shown to be reliable by using the *cry1* gene of *Bacillus thuringiensis* marker as internal control [103].

Allelic discrimination using real time PCR assay developed by Bowers et al., to differentiate *B. mallei* and *B. pseudomallei,* and was named as Burkdiff. This method was developed to differentiate small-nucleotide polymorphisms (SNPs) of *B. pseudomallei* and *B. mallei*. All 469 *B. pseudomallei* isolates tested were shown to contain an allele SNP signature A, while all 49 *B. mallei* isolates were shown to contain an allelic signature C SNP. However, there was no DNA amplification from 390 non-target species tested [104].

7.5.3 Novel Diagnostic Approaches

Specific biomarker-based differentiation between *B. mallei* and *B. pseudomallei* was not successful due to the close relationship between these two pathogens, especially in routine laboratory diagnosis. The Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometric (MALDI-TOF MS) typing technology was used to identify and differentiate burkholderiae using whole bacteria. *B. mallei* showed higher homogeneity than *B. pseudomallei* when using a library generated by 10 *B. pseudomallei* and 17 *B. mallei* strains as reference spectra. This modified MALDI-TOF MS method requires high quality of the reference set to discriminate these two burkholderiae [105].

Despite the high accuracy associated with molecular analysis for target detection for *B. mallei* by PCR methods, many of these techniques require specialized high-cost equipment and remain a challenge for diagnosis in low-income endemic areas. The simple technique for rapid detection of *B. mallei* by a loop-mediated isothermal amplification (LAMP) assay was developed by Mirzai et al. This technique uses

three pairs of primers targeting the *B. mallei* integrase gene and one pair of primers targeting to *is407* gene to amplify and verify LAMP products. The assay results showed and optimal time of 60 min incubation and 22 ng/μl of LAMP products from *B. mallei* strain ATCC 23344 DNA to detect by turbidity change and fluorescence dye with SYBER Green I. The assay's specificity was significantly higher and it was proposed to be an accurate and cost-effective alternative method for *B. mallei* diagnosis in low-income countries and endemic areas in which surveillance is limited [106].

7.6 Concluding Remarks

B. mallei is a mammal-adapted bacterium that has been re-classified numerous times between different genera since its initial discovery. This pathogen remains a potential biothreat and is associated with both military and endemic cases. The association of *B. mallei* with recent outbreaks has led to the classification of *B. mallei* as a re-emerging pathogen, especially in endemic areas. Notwithstanding this re-emergence, this pathogen is correlated with high mortality in both humans and equids. These characteristics linked with *B. mallei* have prompted the need for the development of adequate diagnostics, vaccines, and therapeutics. Nonetheless, the limited understanding regarding the pathogenesis of glanders has hindered the developed of effective countermeasures against this re-emerging threat.

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Burkholderia pseudomallei

8

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8.1 Introduction

Melioidosis is an infectious disease caused by the bacterium *Burkholderia pseudomallei*, a motile, Gram-negative saprophyte commonly found in soil and water in tropical and subtropical areas. *B. pseudomallei* is endemic to areas in South-eastern Asia and northern Australia. Since 1991, cases of melioidosis have been reported in India, Western Asia, China, Sub-Saharan Africa, the Caribbean, and the Americas [1]. Melioidosis presents with non-specific symptoms and clinical signs, and is known as the "great imitator" due to its multifaceted clinical presentation, often leading to misdiagnosis [2]. Infection results in a variety of outcomes, including pneumonia, septicemia, osteomyelitis, abscess formation, and organ failure [3]. Treatment is hampered by an intrinsic resistance to many first line antimicrobial agents resulting in elevated case fatality rates (CFR) [4]. The vast differences in clinical outcomes may be attributed to the availability of critical care services, the virulence of strains endemic to an area, and the underlying health of the patient. Melioidosis is considered an opportunistic pathogen, with high incidence associated with diabetes, excessive alcohol use, chronic renal failure, and lung disease [5].

Melioidosis is thought to be underdiagnosed in many regions due to the non-specific presentation and difficulties in diagnosing the infection. *B. pseudomallei* can cause serious disease in humans and animals; 165,000 people are predicted to be infected annually with a CFR of about 53% [6]. Developing countries such as Thailand and Laos reported epidemics with CFRs exceeding 70% in the absence of treatment [6]. Treatment with ceftazidime and meropenem has

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reduced CFRs to about 40% in these countries [3, 7]. Even with modern medical standards and surveillance programs in Australia and Singapore, these countries still report epidemics with CFRs as high as 20% [8–10]. Melioidosis is difficult to diagnose, lacks an approved vaccine, and has limited treatment options. Together, these factors have warranted the need for medical countermeasures.

8.2 History

8.2.1 First Reported Cases

The word melioidosis comes from the Greek *melis* meaning "distemper", *oid* meaning "resemblance", and the suffix *osis* indicating disease [11]. The first cases of melioidosis were reported in 1912 by pathologist Alfred Whitmore and assistant C. S. Krishnaswami in patients at the Rangoon General Hospital in Burma (modern day Myanmar) [12]. Case 1 was a 40-year old man who had been admitted to the hospital for a fever lasting 7 days. He was a morphine addict, having his thigh covered by injection marks with abscess formation at the site of injection. His lungs showed signs of inflammation dissimilar to lobar pneumonia and tuberculosis. Bacterial culture resulted in non-motile Gram-negative bacilli suggesting the infection was due to *Bacillus mallei* (modern day *Burkholderia mallei*), the causative agent of glanders. Whitmore and Krishnaswami considered case 1 to have glanders, however the patient had been recently released from jail and was not in close contact to horses, making the diagnosis unlikely [12].

Whitmore and Krishnaswami conducted Strauss's guinea pig testicular reaction to determine if the cultured bacterium was *B. mallei* [12]. The Strauss reaction is a skin test used to diagnose *B. mallei* by injecting suspected material intraperitoneally, resulting in a delayed necrotizing inflammation in the testes [13]. Using the culture isolate from case 1, guinea pigs were injected intraperitoneally with the bacterium. Surprisingly, these guinea pigs succumbed to infection within 36 h of injection and showed no obvious signs of inflammation in the testes. Whitmore and Krishnaswami observed acutely inflamed lymph, peri-hepatitis, and injury to the intestines. Bacterial cultures from the guinea pigs were highly motile Gram-negative bacilli suggesting contaminated cultures since *B. mallei* is known to be non-motile. Dissatisfied with this result, Whitmore and Krishnaswami sub-cultured the bacteria from the diseased lung of case 1 and found the fresh subculture to be highly motile but losing motility after a few days in artificial media. This motility defined a new etiological agent. Whitmore proposed the name *Bacillus pseudomallei* due to the nature of infection in the lungs and similarities to glanders caused by *B. mallei*. A subsequent study using guinea pigs found subcutaneous injection of *Bacillus pseudomallei* led to development of lesions at the site of injection and death in 4–5 days [12]. Feeding experiments using contaminated food found guinea pigs to develop sepsis and lesions in the lungs similar to those observed in the human patients [12].

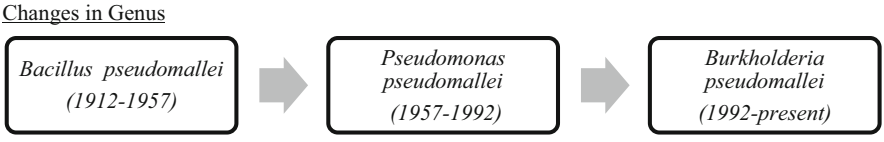
In 1913, a fatal outbreak in laboratory animals occurred at the Institute for Medical Research in Kuala Lumpur, Malaya (modern day Malaysia) [14]. Several

animals exhibited milky discharge from mucosal membranes and died from pulmonary complications. Bacteriologist Ambrose Thomas Stanton and pathologist William Fletcher did not identify the cause of the outbreak to be *Bacillus pseudomallei* until 1917 when Stanton observed similar clinical signs in cases of melioidosis in humans, wild rodents, and a domestic cat. The bacilli isolated from the 1913 outbreak were then identified as *B. pseudomallei.* Stanton and Fletcher further tested the susceptibility of various animals to melioidosis. Guinea pigs, rabbits and rats quickly succumbed to infection when inoculated with fresh cultures. One of three cynomolgus monkeys died from infection when orally fed *Bacillus pseudomallei* due to lesions found in the lungs. Stanton and Fletcher also observed horses that survived *Bacillus mallei* infection were immune to *Bacillus pseudomallei* [14].

8.2.2 Changes in Genus

The sixth edition of *Bergey's Manual* re-classified *Bacillus pseudomallei* as *Pseudomonas pseudomallei* [15]. The genus *Pseudomonas* was described by Migula in 1895 as "cells with polar organs of motility" [16]. This re-assignment to *Pseudomonas* was based on motility, growth on minimal defined media, and use of several organic compounds as energy and carbon sources [17]. The bacterium was also found to metabolize glucose through the Entner & Doudoroff pathway, similar to several aerobic pseudomonads [18].

In 1992 several pseudomonads were reclassified as the genus *Burkholderia*. The *Pseudomonas* genus was divided into five subgroups based on 16S rRNA sequence differences [19]. This change would include the entire homology group II including *Pseudomonas pseudomallei*, *Pseudomonas mallei*, *Pseudomonas cepacia* and four other bacteria [20]. Yabuuchi et al. found these bacteria to be able to metabolize and utilize several disaccharides and polyalcohols, activities not observed by other pseudomonads. Additionally, lipid composition, 16S rRNA sequence, and GC content within the proposed group greatly differed from *Pseudomonas aeruginosa*, the prototypical pseudomonad [20].



8.3 Epidemiology

Melioidosis is considered one of the most neglected tropical diseases. *B. pseudomallei* is endemic to South-eastern Asia and northern Australia. A study published in 1994 estimated the prevalence of melioidosis in Thailand to about 4.4

cases per 100,000 individuals annually [21]. In comparison, the prevalence in northern Australia was about 19.6 cases per 100,000 individuals [22]. The Thai Ministry of Public Health annually reports about 2000–3000 cases per year, a number likely to be lower than the actual number of cases [6, 23]. Two hospitals in Thailand reported the overall cost of US$150,000 and US$450,000 per year associated to bacteremic melioidosis; the economic burden averaging about US$15,000 per fatal case in the region [24].

8.3.1 Spatial Distribution

In an attempt to predict the true global burden, Limmathurotsakul et al. have established a model to determine the global distribution of *B. pseudomallei*. This model can be used to predict melioidosis cases per year based on an index of environmental suitability for the bacteria, population risk factors that may contribute to higher incidence rates, and documented human and animal infections from 1910 to 2014 (Fig. 8.1). The model estimated 165,000 global cases of melioidosis and 89,000 deaths caused by the infection in 2015 [6]. The number of cases predicted by the model is in line with data collected in Australia, Brunei Darussalam, and Singapore (countries with well-funded national surveillance programs) providing power to their predictions. Based on the predictive model, the number of cases of melioidosis are likely to be depressed in 45 countries endemic for *B. pseudomallei* [6]. Southern and South-eastern Asia, including India and Indonesia, is predicted to bear the burden of 44% of all melioidosis infections [6]. In addition, 34 countries proposed to be endemic for *B. pseudomallei* have never reported a locally acquired infection [6].

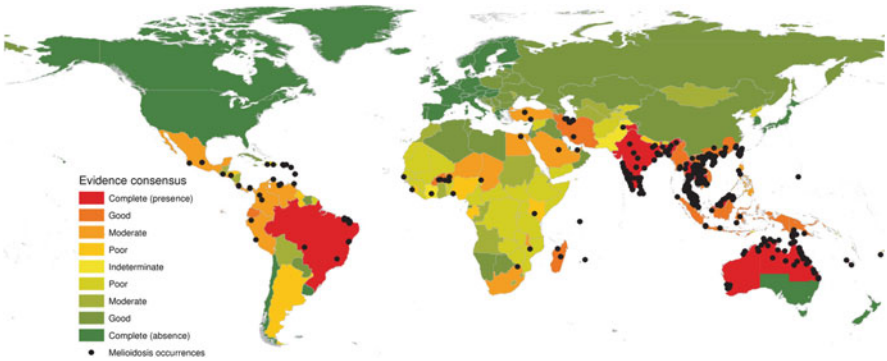


Fig. 8.1 Global evidence consensus and geographic locations of occurrence data from 1910 to 2014. Country coloring is based on evidence-based consensus, with green representing a complete consensus on absence of *B. pseudomallei* and red a complete consensus on presence of *B. pseudomallei*. Black dots represent geo-located records of melioidosis cases or presence of *B. pseudomallei* [6]

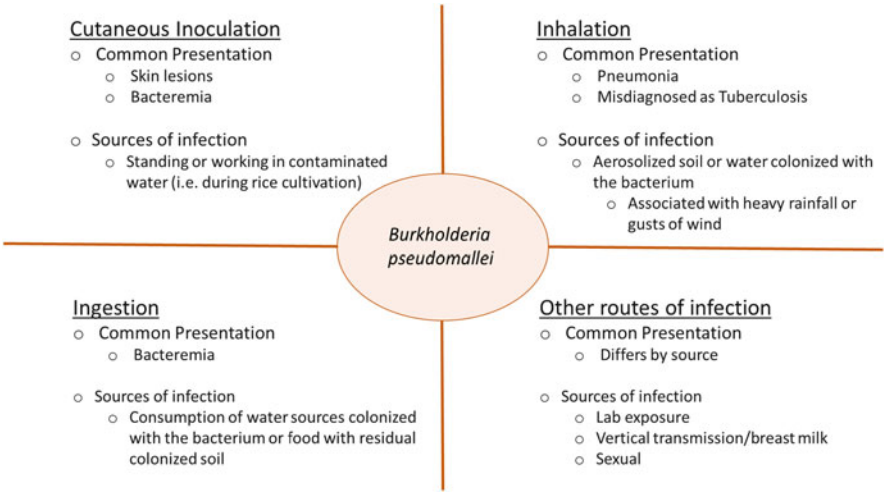
The predictive model also suggested that regions in the United States and Japan, countries thought of as *B. pseudomallei*-free, could support growth of the pathogen. Sporadic cases have been reported in the United States and France, though *B. pseudomallei* has not been identified in these countries [25, 26]. The most probable cause for these cases is traveling to and from endemic regions. The feasibility of supporting growth of *B. pseudomallei* warrants concerns for malicious distribution of the pathogen. Furthermore, diagnosis of melioidosis is unlikely in countries thought to be free of the bacterium.

Whole genome sequencing provides an evolutionary perspective on strain origins. It is believed that the first isolate of *B. pseudomallei* originated in Australia, with a division of Australian and Asian strains during glacial periods of history [27]. Biogeographic distribution of clinical isolates may be a contributing factor to disease outcome. A recent study mapped genetically distinct isolates of *B. pseudomallei*, found in Asia and Australia, by sequence typing (ST) [28]. The results indicate that although distinct sequence type strains had been segregated throughout the sample history, a limited number of strains associated with Asia have been introduced to Australia and are increasing in prevalence. However, it is unclear if the Asian strains, novel to Australia, are driving recent infection patterns because of a higher or differential virulence potential [29].

8.4 Mode of Transmission

While rare cases of human-to-human transmission have been reported, infection is more commonly acquired from the environment, as the organism is prevalent in the water and soil in endemic regions [30, 31]. As a soil-borne bacterium, *B. pseudomallei* can persist in the soil for years, with optimal soil for survival found to be rich in iron, with an acidic pH and water content of 40% [32, 33]. Other soil factors that have been found to enhance survival of *B. pseudomallei* include high salinity, rich in clay, and manipulation by man [6]. Soil disruption by rain, wind, or animals (including humans) results in the aerosolization of the organism allowing for a broader distribution in the water or air. The rainy season in endemic areas results in an influx of inhalational cases of melioidosis, likely due to changes in soil chemistry along with aeration of the organism in the soil [6, 34]. Monsoon seasons and heavy rainfall have also been associated with inhalational melioidosis in areas with high population densities (such as Singapore), and therefore lower concentrations of exposed soil, suggesting that moisture or humidity may contribute to the aerosolizing of the bacteria [35]. Likewise, a study from Taiwan supports that the pathogen can be aerosolized and that direction of winds can predict the locale of instances of melioidosis [36]. Location of urban areas, in relation to environmental pools of the bacterium, may contribute to rates of infection, as seen in the areas surrounding Taiwan and Darwin, Australia [36, 37]. There is no clear rule between population density and rates of melioidosis infection, as in northern Australia and Singapore rates are higher in urban areas, whereas in Thailand the rural population has higher rates of infection [38]. In

Table 8.1 Modes of transmission and associated disease outcomes



endemic regions, agricultural workers commonly (5–20%) have antibody titers to the bacterium, however may not present with clinical signs [39]. When clinical signs are present, melioidosis frequently results in bacteremia, pneumonia, or cutaneous or internal abscesses. With ambiguous clinical signs, an initial diagnosis can rely to occupation, medical history, or travel history [40, 41]. The source of infection can be a great predictor of how the disease progresses (Table 8.1), however, the overall case fatality rate associated with the disease when left untreated can exceed 70% [6, 41].

8.4.1 Cutaneous Inoculation

Exposure, through an open wound, to *B. pseudomallei* in the soil or water is thought to be a main source of infection in endemic regions. Epidemiological studies have found that correlations exist between activities during which patients are exposed to the soil or water and positive culture results [31, 33]. Cutaneous inoculation can result in ulcers on the skin which may lead to bacteremia [42]. Colonization of local water supplies or standing pools of water allow for an easily accessible source of inoculation through open skin abrasions. The cultivation methods of a main agricultural crop of endemic areas of South-eastern Asia (rice paddies) along with prevalence of the bacteria in the soil and water, places rice farmers at elevated risk for cutaneous infections.

8.4.2 Ingestion

As an environmental bacterium, *B. pseudomallei* is routinely found in the water supply of endemic regions, making ingestion an accessible route of infection. A recent study in Thailand indicates that in endemic areas 12% of household water supplies, either from tap water or bore holes, are contaminated with *B. pseudomallei* [33]. In rural areas of northern Australia, untreated bore holes are a common source of water. Exposure to *B. pseudomallei* through bore hole water is seasonal, with 33% and 20% of sampled water supplies testing positive for the pathogen in the wet and dry season, respectively [37]. Patient outcomes associated with ingestion of contaminated water or soil result in a higher incidence of bacteremia than pneumonia [31]. It is not currently understood if ingested *B. pseudomallei* can colonize the gut of humans. However, fecal and/or rectal samples from 10% of hospitalized melioidosis patients in Thailand were positive for the organism, indicating that the organism passes through the gut of some patients [43]. The organism is not believed to be a part of the normal GI microbiota; therefore, the presence of the bacteria in fecal samples suggests some involvement with disease progression [40].

8.4.3 Inhalational

A diagnosis of melioidosis through an inhalational route of infection may be most relevant in response to a bioterrorism event, and necessitates meeting 5 criteria: (1) respiratory symptoms, (2) sepsis, (3) a documented infiltrate in the lung, (4) no sign of cutaneous inoculation and inhalational exposure, and (5) isolation of the pathogen from the patient [40]. As in the case of cutaneous inoculation and transmission through ingestion, inhalation of *B. pseudomallei* is associated with the exposure to soil or water colonized with the bacteria. In terms of clinical outcomes, inhalation of bacteria, associated with water or soil aerosols, has a higher correlation with pneumonic cases of disease than bacteremia [31, 40, 44]. Despite the origin of the bacteria, from a natural environmental reservoir or contamination from a clinical isolate, aerosolization results in an elevated risk for the introduction of *B. pseudomallei* to the lung, resulting in high levels of morbidity and lethality.

8.4.4 Other Routes of Transmission

Although the majority of cases are thought to be due to inhalation, ingestion, or cutaneous inoculation, other routes of infection have been documented. Human-to-human transmission has been recorded through the ingestion of infected breast milk [42], vertical transmission [45], and sexual transmission [30]. In addition, infection through laboratory exposure has also been reported [30].

8.5 Clinical Manifestations

8.5.1 Clinical Signs and Outcomes

In the early 1900s, Whitmore suggested melioidosis to be an infection of morphine-addicts. However, the disease has been observed in healthy individuals [12]. *B. pseudomallei* has been suggested to be an opportunist pathogen. Risk factors for melioidosis include: diabetes mellitus, chronic liver or kidney disease, alcohol abuse, long-term steroid use, hematologic malignancy, neutropenia or neutrophil dysfunction, chronic lung disease, thalassemia, or forms of immunosuppression (Table 8.2) [5]. Diabetes mellitus is the major associated condition with acute melioidosis, but a correlation was not found between diabetes and chronic melioidosis [38, 50]. Liver and kidney disease, along with heavy alcohol use, are also major risk factors for melioidosis [5, 38]. Diabetes and liver and kidney disease suggest the impairment of the innate immune response contributes to the pathogenicity of *B. pseudomallei* [38]. However, individuals with human immunodeficiency virus 1 (HIV-1) infection are not at a higher risk for melioidosis compared to healthy

Table 8.2 Treatment, risk factors, resistance and diagnosis of melioidosis [5, 46–49]

Treatment

Recommended treatment by the US Centers for Disease Control and Prevention:

- First 10-14 days post-exposure:
  - Ceftazidime
    OR
  - Meropenem
- Following 3 to 6 months:
  - Trimethoprim-sulfamethoxazole
    OR
  - Doxycycline

Intrinsic Antibiotic Resistance

Burkholderia pseudomallei is intrinsically resistant to the following antibiotics:

- Aminoglycosides
- Ampi-/amoxicillin
- Cephalosporins
- Erythromycin
- Gentamicin
- Macrolides
- Penicillin
- Polymyxins
- Rifampicin

Risk Factors

Risk Factors for melioidosis include:

- Diabetes mellitus
- Chronic liver or kidney disease
- Alcohol abuse
- Long-term steroid use
- Hematologic malignancy
- Neutropenia or neutrophil dysfunction
- Chronic lung disease
- Thalassemia
- Other forms of immunosuppression

Diagnosis

Gold Standard:

- Bacterial culture
- Gram stain

Secondary Methods:

- Mass spectrometry
- Polymerase Chain Reaction
- Immunoassay

individuals [51]. The country in which a patient contracted and was diagnosed with melioidosis may contribute to clinical outcome as much as the patient's prior medical history. The vast differences in clinical outcomes for melioidosis patients may be attributed to the resources available to the healthcare provider, as well as *B. pseudomallei* strains colonizing the country of transmission [6, 28].

Melioidosis patients present with a broad array of clinical signs, from cutaneous lesions to pneumonia and bacteremia. The site of colonization within the host and outcome may, in part, be attributed to the mode of transmission. Cutaneous inoculation, through a skin abrasion, can cause skin ulcers which may lead to bacteremia. Interestingly, ingestion of *B. pseudomallei* results in a higher incidence of bacteremia than pneumonia [31, 42]. Transmission via inhalation commonly results in pneumonia (~50% of cases), with the pathogen disseminating from the lung, resulting in an outcome of septicemia, in fewer cases [40, 44]. While the overall percentage of cases with serious disease manifestations, such as pneumonia or bacteremia, is high, pediatric cases that result in pneumonia and bacteremia is lower than those seen in adults, at 20% and 16%, respectively [42]. Interestingly, children who tested positive for melioidosis had fewer risk factors for the disease, such as diabetes, then melioidosis-positive adults. The correlation between risk factors for disease and disease manifestation suggests that having risk factors may result in more serious disease outcomes. Soft-tissue abscess formation is a less common but notable outcome of melioidosis and is independent of pneumonic status (Fig. 8.2) [31, 44]. The complexity and rapid progression of the disease can be

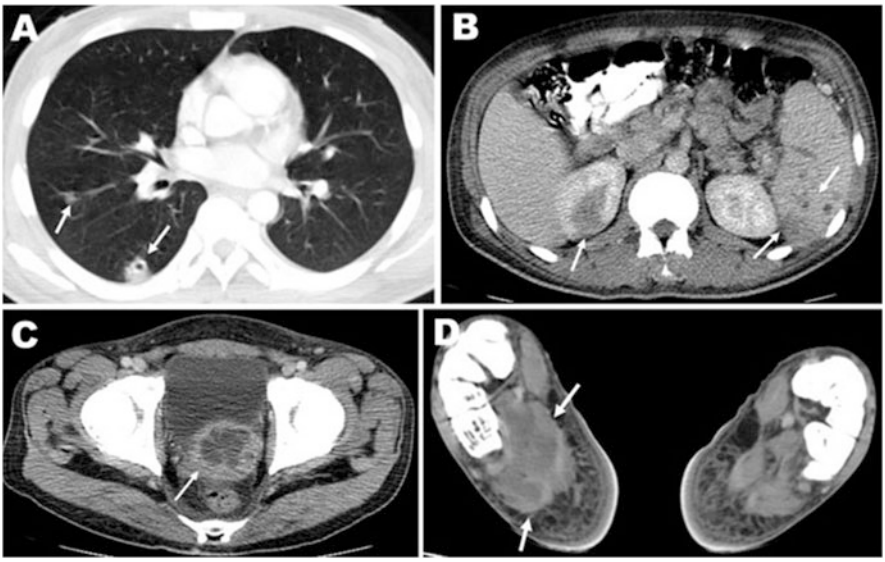


Fig. 8.2 Abscess formation in an agricultural worker from Thailand—Computed tomography images showing multiple abscesses in the right lung (**a**), spleen and upper pole of the right kidney (**b**), prostate gland (**c**), and plantar aspect of the right foot (**d**) (arrows) of the patient [52]

demonstrated by the seemingly short incubation period, an average of 9 days, to the range of severe disease outcomes present at the initial disease presentation [38, 52].

8.5.2 Pathophysiology

Melioidosis is known as the "great imitator" because patients present with non-specific clinical signs that can mimic more common infections. Clinical presentation can range from asymptomatic to chronic infections (Table 8.1). *B. pseudomallei* has an incubation period of 1–21 days, averaging 9 days [5]. Acute manifestation can result in asymptotic infections or localized skin abscesses [53]. Pulmonary melioidosis is commonly misdiagnosed as tuberculosis [54]. Both melioidosis and tuberculosis present similar leukocyte and neutrophil counts, erythrocyte sedimentation rate (ESR), and blood glucose levels [54]. Other clinical manifestations include septicemia, abscess formation, osteomyelitis, and organ infections [55]. Abscess formation and organ failure is most common in lungs, liver, spleen, and prostate. Melioidosis is responsible for 20% of community-acquired septicemias in northeastern Thailand [56].

B. pseudomallei can evade the host immune system by escaping the phagosome of phagocytic cells or inducing invasion using the type III secretion system of non-phagocytic cells [57, 58]. When internalized, the bacterium can utilize cellular actin to move within the cell (Fig. 8.3). Actin-mediated motility facilitates cell-to-cell spreading of *B. pseudomallei*, resulting in multinucleated giant cell (MNGC) formation [60]. This can lead to apoptosis or membrane disruption followed by bacterial dissemination [60]. During an infection, *B. pseudomallei* has been shown to modulate alterations of host metabolites such as calprotectin, a factor that could be utilized in a diagnostic assay [61]. Recent studies have identified a panel of host metabolites that are upregulated following *B. pseudomallei* infection [62]. Plasma collected from 22 septicemic melioidosis patients showed distinct metabolomic profiles, compared to sepsis caused by other bacteria and a control group of healthy individuals (Fig. 8.4). Host cells use pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and Nod-like receptors, to recognize an infection. Most Gram-negative bacteria are recognized by TLR4; however, TLR4 knockout mice infected with *B. pseudomallei* exhibit a similar phenotype to wildtype [63]. TLR2, characterized as a Gram-positive bacterium recognition receptor, is activated by lipopolysaccharide (LPS) on the surface of *B. pseudomallei* [63]. This activation is reported to impair the host response to sepsis [63]. Cells infected with *B. pseudomallei* have been shown to express low levels of inducible nitric oxide synthase (iNOS) and tumor necrosis factor α (TNF-α), important for macrophage activation [64]. Furthermore, seropositive whole blood taken from patients in northeastern Thailand had increased concentrations of the anti-inflammatory cytokine interleukin-10 (IL-10) when co-cultured with *B. pseudomallei* in vitro [65]. Notably, IL-10 inhibits production of pro-inflammatory cytokines TNF-α, interferon-γ (IFN-γ), and interleukin-6 (IL-6) [65]. Increased levels of IL-10 in blood may dampen the immune response, therefore leading to susceptibility to septicemia.

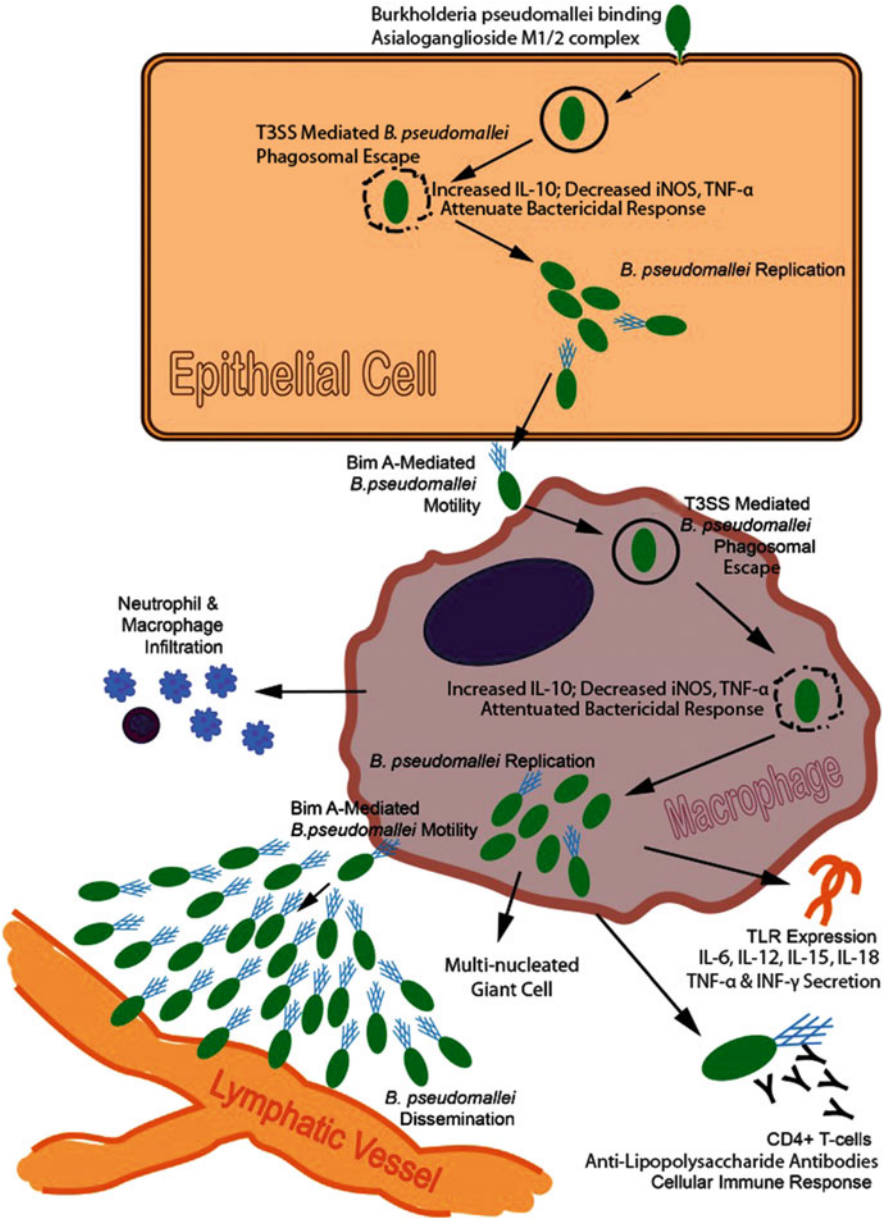


Fig. 8.3 Mechanisms of internalization by *Burkholderia pseudomallei. B. pseudomallei* uses host actin for intracellular movement and invasion of neighboring cells, leading to the formation of multinucleated giant cells. The host exhibits innate and adaptive immune responses; however, the pathogen can attenuate the host immune system by modulating cellular host responses (modified from Bocan et al.) [59]

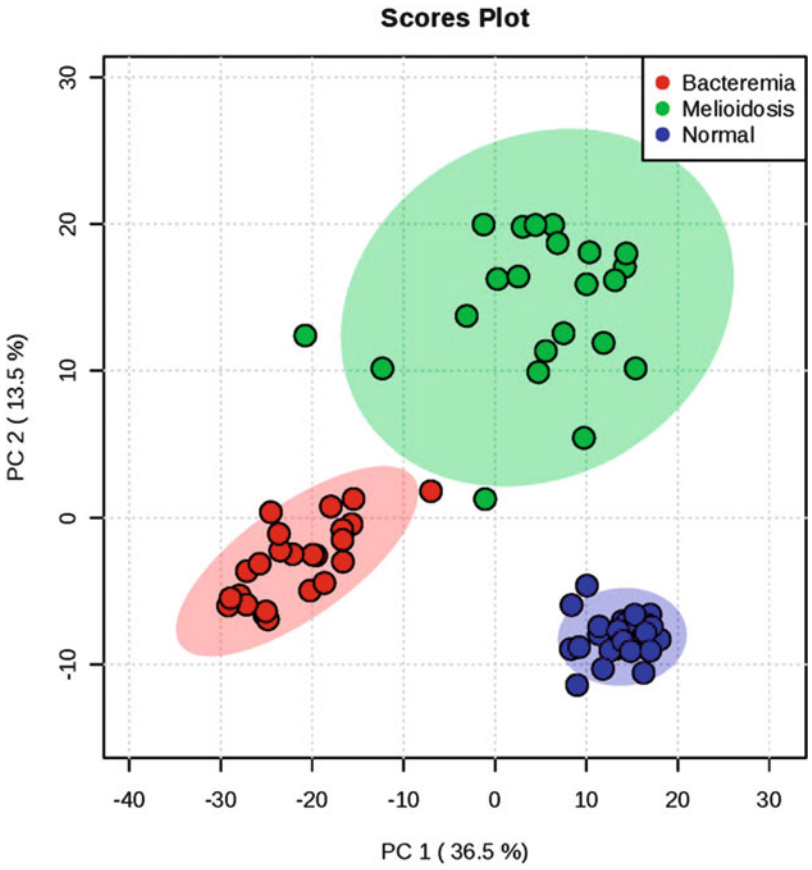


Fig. 8.4 Principal component analysis of metabolomic profiles from 76 patient samples. Data collected by ultra-high performance liquid chromatography- quadrupole time-of-flight mass spectrometry (UHPLC-QTOFMS) showed patients with septicemic melioidosis have a distinguishable pattern compared to sepsis caused by other bacteria and a control group. Twelve host metabolites were identified to be upregulated in melioidosis patients and may be used as biomarkers of septicemic melioidosis [62]

8.6 Molecular Aspects of Disease

B. pseudomallei has a large genome that drives not only the virulence of the organism but also allows for survival in diverse environments. Analysis of the 7-Mb genome indicates that there are over 4300 coding DNA sequences (CDSs), a number that is dependent on the strain. Using a comparative genomics approach, the core genome of *B. pseudomallei* indicates 2339 conserved CDSs [66]. Factors contributing to the pathogenicity of *B. pseudomallei* include genomic islands and

prophages, intrinsic antibiotic resistance mechanisms, multiple polysaccharide encoding operons, and morphological variability.

8.6.1 Genomic Islands and Prophage

While *B. pseudomallei* is distinct from its taxonomic near neighbors, there is a high degree of diversity among strains, much of which can be attributed to genomic islands (GI) and prophages. These segments of DNA maintain characteristics such as altered GC content, distinguishing the GI from surrounding sequence. GIs vary in length and commonly contain factors that allow for a fitness advantage, such as pathogenicity islands or metabolic islands [67]. Although the genome sequence as a whole is conserved, genomic islands are more easily transferable from strain to strain, introducing population diversity. A study using comparative genomics to assess differences between strains with soil or clinical origins determined that there are at least 15 GIs contained within the pan-genome of *B. pseudomallei* [68]. While the combination of GIs present can be used as a predictor of strain origin, it cannot be used to predict virulence [68]. Many of the GIs include genes involved in metabolism or cell structure, allowing for specialization of each strain [68]. Prophages contribute to a substantial fraction of chromosomally harbored genomic islands [69]. These sequences are marked by phage attachment sites and tRNA-encoding genes [69]. One study assessed that phage-like or prophage-encoded DNA contributed to interspecies diversity when comparing the genomes of *B. pseudomallei* and closely related species [69]. Interestingly, no evidence of prophages was found in the genome of the closely related species, *B. mallei* [69].

8.6.2 Colony Morphology

A characteristic of *B. pseudomallei* colonies is the polymorphic profile. Upon examination of growth of a single strain of *B. pseudomallei* on a solid surface, it is common to find multiple colony morphologies (Fig. 8.5). The array of phenotypes may be attributed to environmental stresses found in different niches, as the bacterium alters its cellular dimensions in response to compounds found within the environment [32]. Strains of *B. pseudomallei* produced up to 24 morphological variants when plated on Ashdown agar [70]. Experiments assessing environmental and host effects on these strains suggest the number of morphological variants were narrowed in response to various media and when passaged in vivo [70]. Metabolomic analysis indicates that *B. pseudomallei* exposed to in vivo growth conditions is driven to usage of a more succinct set of amino acids than strains cultured in vitro, a change that coincides with the narrowing of morphological phenotypes expressed [70]. In addition, proteomic assessment of cells from colonies with different morphologies found changes in protein levels necessary for carbohydrate usage, metabolism, and factors attributed to persistence in the host (i.e., adhesion and host-cell invasion) [71]. Expression of antigenic variants of LPS resulted in changes in the

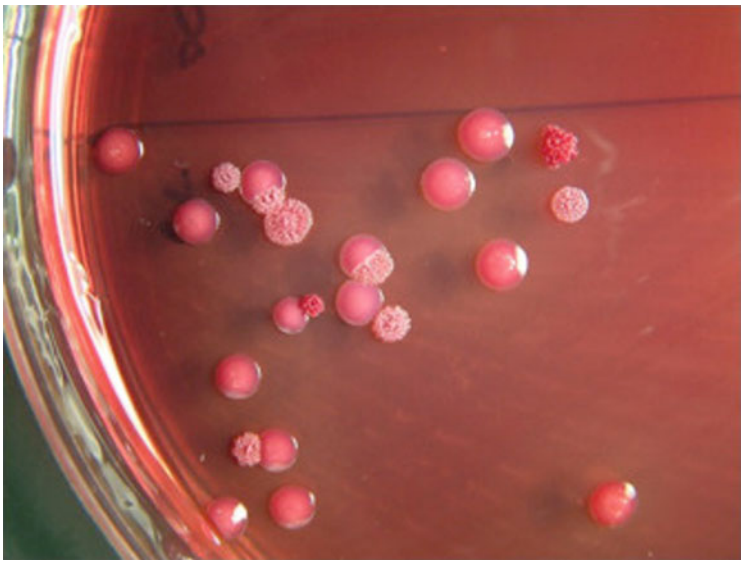


Fig. 8.5 *Burkholderia pseudomallei* exhibits a variety of colony morphologies when grown on Ashdown agar at 37 ° C for 4 days [44]

percentage of cells internalized in monocyte internalization studies; changes in cell surface localized proteins and carbohydrates may subsequently result in increased survival in the host [72]. The variability of cell morphology appears to be an adaptive mechanism for environmental changes [73].

8.6.3 Antibiotic Resistance

The US CDC recommends an intensive, two-phase treatment for patients diagnosed with melioidosis. *B. pseudomallei* is intrinsically resistant to many antibiotics, including some considered last resort in treatment for other bacterial infections [74]. Multiple mechanisms dictate the resistance of *B. pseudomallei*, including efflux of the drug, a minimal number of penicillin-binding protein encoding genes, and expression levels of β-lactamases [75]. While resistance-encoding genes are commonly found on plasmids, the resistance-encoding genes of *B. pseudomallei* are chromosomally encoded, indicating the gene products may enhance the fitness of the organism [75]. The established core-resistome of *B. pseudomallei* includes resistance to gentamicin, multiple β-lactams, rifampicin, and erythromycin (Table 8.2). In most cases, *B. pseudomallei* remains susceptible to ceftazidime (CAZ), a broad spectrum cephalosporin, making CAZ the first line of treatment for most cases of melioidosis [76]. However, resistance to CAZ is on the rise and molecular analysis of in vivo-induced resistant isolates indicates mutations in the *penA* gene [76]. In addition, resistance to doxycycline, a second antibiotic commonly used to treat

melioidosis, has also been documented. A recent study from a group in northern Australia used comparative genomics to assess the genetic differences between two strains of *B. pseudomallei* isolated from a patient pre- and post-treatment with doxycycline. The authors describe point mutations in two loci, one in the repressor of an efflux pump and the second in a S-adenosyl methionine (SAM)-dependent methyltransferase gene [77]. Despite what appears to be movement toward a critical loss of effective antibiotics to treat melioidosis, preliminary trials of a recently developed fluoroquinolone, finafloxacin, with activity in low pH environments has been found to be effective against *B. pseudomallei* in a mouse model of infection [78]. The intrinsic antibiotic resistance and development of resistance to remaining antibiotics warrant the need for alternative treatment options for melioidosis.

8.6.4 Capsular Polysaccharide (CPS)

B. pseudomallei forms a capsule composed of polysaccharides to effectively inhibit phagocytosis and increase survival in the mammalian host. Using genomic approaches, four independent operons (CPS I–IV) were identified within the *B. pseudomallei* genome, with the aforementioned phenotypes attributed to CPS I [79–81]. Transcriptional profiling indicated that the CPS I operon is up-regulated in the mammalian host; CPS III is expressed in water and down-regulated when grown in the presence of normal human serum [81]. Though the spatial expressions of CPS II and CPS IV have not been reported, the presence of four large CPS-encoding operons suggests variable structures in the capsule dependent on environmental conditions. The in vivo capsule (CPS I) has been found to have multiple roles in infection. These include limiting complement deposition and contributing to biofilm development, a structure that may play a role in the pathogen's ability to cause a relapsing infection [82, 83]. In addition, CPS I-deficient strains are attenuated in both the ability to colonize and disseminate from the lungs in mouse models of infection, establishing the capsular polysaccharide as a virulence factor [84, 85].

Opsonization of bacteria increases rates of phagocytosis by phagocytic cells. In a recent study assessing the effects of CPS I on opsonization and phagocytosis in vitro, encapsulation decreased opsonization of *B. pseudomallei* but did not have significant effects on phagocytosis or survival within the phagocyte [86]. While CPS did not contribute positively to survival in the in vitro study, antibodies to CPS do protect the host in a mouse model of infection—a finding that is enhanced when CPS antibodies were mixed with LPS (lipopolysaccharide) antibodies [87]. Due to the lack of a vaccine against melioidosis, an antibody-based therapeutic targeting the carbohydrates produced by *B. pseudomallei* may prove to be an alternative to increase the survival rate of infected people.

8.7 Mechanisms of Pathogenesis

The diversity of the protein encoding genes expressed by *B. pseudomallei* allows the organism to survive both in the environment and a mammalian host. A recent proteomic study described how proteins are differentially produced by an environmental isolate pre- and post-host adaptation. The study indicated that of the proteins identified, greater than 10% were differentially produced, suggesting the bacterium to have evolved separate mechanisms for intra-mammalian and environmental survival [88]. Likewise, *B. pseudomallei* may produce different proteins or carbohydrates that are critical for infection at distinct foci within the host. *B. pseudomallei* is capable of surviving extracellularly, attaching to and invading non-phagocytic cells, or surviving within and escaping from phagocytic cells.

8.7.1 Cell Attachment and Invasion

A critical step in the host-bacterial interaction is the attachment of a bacterium at a host cell receptor, leading to bacterial uptake. The interaction between *B. pseudomallei* and non-phagocytic cells has not been well-defined; however, evidence suggests that a variety of bacterial macromolecules may play analogous roles in this process. Two such proteins, termed BoaA and BoaB, are homologous and are predicted to share a domain that is a member of the Oca family of autotransporters and adhesions. *B. pseudomallei* strains with *boaA* and/or *boaB* gene deletions exhibit a defect in adherence to epithelial cell lines. Furthermore, when the genes are exogenously expressed in *E. coli*, the bacteria exhibit an increase in adherence to epithelial cells [89]. While the deletion of *boaA* and *boaB* genes did not eliminate adherence, there was substantial adherence reduction, indicating that the genes are not the sole adherence factors expressed by *B. pseudomallei*. A second family of genes that have been demonstrated to contribute to host cell adherence by *B. pseudomallei* are the type IV pilus-encoding genes. A deletion of the *pilA* gene resulted in a reduced percentage of bacteria that adhere to human epithelial cell lines compared to the parental strain (K96243) [90]. The reduction in adhesion of the *pilA* mutant may be a contributing factor to the attenuation of the strain in an intranasal mouse model of infection [90]. However, in the same study, the mutant strain did not demonstrate a defect in virulence in mice inoculated via an intraperitoneal route, suggesting different adhesion factors for colonization of varying host sites or cell types [90].

Some evidence suggests a mechanism for adhesion is via the asialoganglioside M1/2 complex. Pre-treatment of *B. pseudomallei* with purified carbohydrates asialoganglioside M1 (aGM1) and asialoganglioside M2 (aGM2) limited subsequent attachment to primary human epithelial cells [91]. Effects of aGM1 were dose-dependent but strain-independent, indicating that *B. pseudomallei* adhesion occurs, in part, via the aGM1/2 complex [91]. This study confirmed previously published data, obtained using thin layer chromatography, that *B. pseudomallei* interacts with aGM1 and aGM2 [92]. Similar experiments with the related bacterium, *Pseudomonas aeruginosa*, indicated that binding of bacteria to the aGM1 receptor is dependent

on type IV pili [93]. These studies suggest one mechanism for adherence, and therefore subsequent intracellular survival; however, due to the complex nature of the *B. pseudomallei*-host cell interaction, it is likely only one of many mechanisms.

8.7.2 Secretion Systems: Type III and Type VI

Type-three secretion systems (T3SS) are established virulence factors for a variety of pathogens, including *B. pseudomallei*. The effector protein BipC has been demonstrated to be an integral component of the adhesion, invasion, and survival processes. Deletion of *bipC* not only resulted in a decline in bacterial intracellular survival, but more generally the mutant strain was less virulent in a mouse model of infection [94]. Mechanistically, the effector protein binds the host actin protein; BipC can polymerize the host protein in vitro, similar to the activity of another *B. pseudomallei* T3SS protein, BimA [95–97]. By modulating the polymerization/depolymerization of actin, the bacterium is able to affect the integrity of the host cell, allowing for intra- and/or intercellular movement. A recent study examining the ability of a library of *B. pseudomallei* mutant strains to survive and compete in a respiratory model of infection highlights the fitness deficit of *bipC* mutants and multiple other T3SS mutants [84]. A similar survival deficit was found in type-six secretion system (T6SS) cluster 5 mutants in a respiratory model (Fig. 8.6),

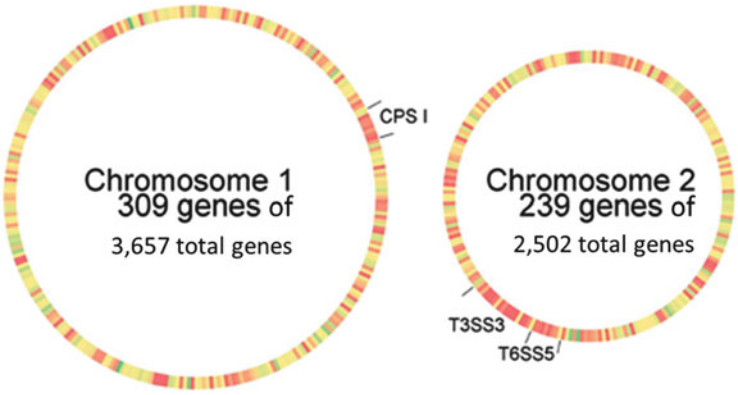


Fig. 8.6 Proximity heat map of genes required for respiratory melioidosis. A Tn-Sequence library (88% coverage) was screened for necessity for infection in a C57BL/6J mouse respiratory model of infection. Using a 15-fold cut off relative to the input pool, 548 genes were identified as necessary for infection in the lung. Identified genes were mapped to each chromosome (309 genes on chromosome 1 and 239 on chromosome 2) with colors indicating proximity to next identified gene, red = 1 gene distance and green = a > 70 gene distance. Three largest clusters of virulence determinant encoding genes identified are the CPS I cluster, T3SS cluster 3, and the T6SS cluster 5. Figure modified (total annotated gene numbers per chromosome for strain 1026b added) from Gutierrez 2015 [84]

suggesting a genetic redundancy among the vast array of virulence determinants produced by *B. pseudomallei* that contribute to its pathogenicity.

The spread of *B. pseudomallei* between host cells (phagocytes or non-phagocytes) is a critical factor in the dissemination of the pathogen (Fig. 8.3). The bacteria utilize several bacterial and host cell factors for translocation, including mobility, host cell membrane disruption, and host cell fusion. The genome of *B. pseudomallei* harbors 6 complete clusters of the T6SS genes (T6SS 1–6), once again suggesting evolutionary tracts, specific to environmental niche, may have merged in a single organism [98]. Distinct domains of the T6SS protein homolog, VgrG, have been demonstrated to be integral to the mechanisms of cell-to-cell fusion, as well as secretion of other T6SS effector proteins [99, 100]. One additional T6SS effector protein that is critical during infection is Hcp-1; mutations in the protein encoding gene result in decreased cytotoxicity in phagocyte infection studies and contribute to the formation of multinucleated giant cells [100]. Furthermore, the Hcp-1 protein elicits an immune response within the host [101]. Together, these findings indicate that both T3SS and T6SS contribute to pathogen survival in the host.

8.8 Countermeasures to Biological Terrorism and Warfare

Burkholderia pseudomallei is classified as a Tier 1 Select Agent by the United States Departments of Agriculture (USDA) and Health and Human Services (HHS). *B. pseudomallei* is one of a handful of etiological agents that overlaps both departments due to the threat to humans, animals, and plants. It is unclear how colonization of plants contributes to the spread of zoonotic and human cases [102]. With current models predicting environmental suitability for the bacterium, preemptive measures can be taken to prevent the malevolent use of *B. pseudomallei*.

8.8.1 Biothreat

Accounts of the use of *B. mallei* on enemy cavalry suggests the potential for *B. pseudomallei* as an agent of biological warfare [103]. *B. pseudomallei* has not been used as an agent of biological warfare, but the bacterium was studied as a possible source material for bioweapon construction by the United States of America, Soviet Union, and possibly Egypt [103]. Historically, members of the military have exhibited higher incidence of melioidosis, potentially due to exposure in the field. Several hundreds of French and American soldiers deployed to Vietnam during the Indochina War (1946–1954) and Vietnam War contracted melioidosis [104, 105]. Likewise, sporadic cases of melioidosis were observed in American and Japanese soldiers post-World War II [106, 107]. Finally, trained armed forces in Singapore have a four-fold higher incidence of infection compared to the general population [103, 108].

The prevalence of *B. pseudomallei* infection outside of endemic areas remains low; however, the sporadic cases that have been reported signify that a diagnosis of melioidosis is not without precedence. While the source of the majority of cases reported in areas deemed *B. pseudomallei*-free have been identified (e.g., foreign travel, occupational exposure, or the import of contaminated soil), the source of a number of cases remains unresolved [6, 30]. The lack of consideration of melioidosis in non-endemic areas may delay diagnosis and reporting of an intentional release of the pathogen. It has been suggested that tightening the reporting regulations for melioidosis infections outside of endemic regions may reduce the chance of use of *B. pseudomallei* as a biothreat or at least limit the associated morbidity.

8.8.2 Countermeasures

The gold standard for diagnosing melioidosis is a culture-positive patient sample, a difficult standard for a pathogen with a low bioburden [109, 110]. Upon diagnosis, the patient undergoes extensive antibiotic treatment, including intravenous antibiotics. Without treatment, the patient is at risk for a number of complications, including (but not limited to) pneumonia, sepsis, neurological infection, and internal abscess formation—outcomes that have long-term repercussions and high case fatality rates [111]. Mitigation of risks associated with the disease and the spread of melioidosis may start with strengthening the surveillance networks around the world, not just in endemic regions [30]. The disease potential associated with *B. pseudomallei* necessitates rapid assessment of infection status to achieve the best possible clinical outcome. Efforts are underway to develop rapid, cost-effective diagnostics that are accessible to rural, infrastructure-limited areas of the world. Low complexity diagnostics, such as the lateral flow immunoassay (technology renowned for its ease of use in the home pregnancy test), have been applied to the detection of *B. pseudomallei* polysaccharides; a version of that test has successfully moved out of the basic research phase of testing to assessment in endemic regions [112, 113]. Molecular assays, both real-time PCR- and isothermal amplification-based, are also in stages of development and optimization for the detection of *B. pseudomallei*. T3SS-associated genes are specific and sensitive targets for detection of *B. pseudomallei* using molecular assays in a variety of clinical samples [114–116]. In addition, host biomarkers, specific to melioidosis patients, are being identified to not only diagnose infection but also assess disease progression [61, 62].

In addition to the development of diagnostics that will result in earlier administration of treatment, work is underway to meet the need for a vaccine to prevent melioidosis. Several groups have reported positive results for candidate vaccines using mouse models of infections. Many of the vaccine variants are based on formulations that include one or more polysaccharides produced by *B. pseudomallei* [117–121]. While pathogen-specific proteins are included in some of the vaccine formulations, it is the polysaccharide that is the common element, indicating that capsular polysaccharide or lipopolysaccharide of the *Burkholderia* species may be a key to unlocking the evasive vaccine for this devastating pathogen.

A final issue that should be addressed in the discussion of strategies to alleviate the morbidity and lethality of *B. pseudomallei* infection is the reduction or eradication of the bacterium from the environment of endemic regions. As *B. pseudomallei* is a ubiquitous soil organism, such eradication would be a challenge, however, studies of the terrestrial microbiome have revealed that *Burkholderia multivorans* inhibits the growth of *B. pseudomallei* [122]. *B. multivorans* is a soil pathogen that is stable in the environment at a larger range of pH and salinity than *B. pseudomallei*; however, it has also been found to colonize the lungs of cystic fibrosis patients [122, 123]. While the organism itself may not be a feasible option to inhibit *B. pseudomallei* in the environment, it does produce a compound that is already ubiquitous to soil that could be used to purge *B. pseudomallei* from the agricultural and urban areas in which it is most problematic.

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Coxiella burnetii: Hiding in Plain Sight

9

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9.1 Introduction

9.1.1 Historical Significance

Coxiella burnetii, a Gram-negative intracellular pathogen, is the causative agent of the zoonotic disease Q, or "query", fever. This disease was first observed in 1935 in nine abattoir workers from Brisbane, Australia, who all presented with a febrile illness [1]. Although the identity of the organism responsible for the disease was unclear, injection of guinea pigs with blood from these patients reproduced the clinical signs and caused enlarged spleens [1]. Splenic tissue from infected guinea pigs, provided by Edward Derrick, allowed Frank Macfarlane Burnet and Mavis Freeman to isolate a filterable "rickettsia-like" organism [2]. Almost simultaneously, during a study of Rocky Mountain spotted fever in Montana, USA, an unknown agent was discovered in ticks collected from Nine Mile, Montana. When these ticks were allowed to feed on guinea pigs, a febrile illness developed that did not replicate Rocky Mountain spotted fever [3]. The Nine Mile agent was filterable and able to be serially passaged in guinea pigs but could not be grown axenically (host cell-free) [3]. Linking the Nine Mile and Q fever organisms occurred in an unexpected manner after a laboratory-acquired infection of the Nine Mile agent resulted in clinical signs similar to Q fever and successive cross-protection studies confirmed that these two organisms were the same pathogen [4, 5]. The pioneering work of both Burnet and Cox to identify the organism responsible for Q fever was venerated by the classification of the causative agent with the genus "*Coxiella*" and species "*burnetii*". *Coxiella burnetii* was placed into the *Rickettsiaceae* family, despite not displaying

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characteristics typical of rickettsiae [6]. Many years later, phylogenetic analysis of the 16S rRNA-encoding gene sequence of *C. burnetii* revealed a much closer relationship to the order *Legionellales*, resulting in the reclassification of the bacteria to the phylum *Proteobacteria*, class γ-*Proteobacteria*, order *Legionellales*, family *Coxiellaceae* [7]. This is quite distant to the *Rickettsiaceae* family belonging to the order *Rickettsiales*, class α-*Proteobacteria* in the phylum *Proteobacteria* [8].

The severe public health and economic devastation that follows a Q fever epidemic has recently been highlighted with the largest ever reported natural outbreak of Q fever occurring in the Netherlands. Such outbreaks highlight the importance of sustained investment into *Coxiella* research due to the wide-ranging impact of this disease in society in addition to its potential for weaponization.

9.1.2 *C. burnetii*: The Bacterium

C. burnetii is a pleomorphic coccoid bacterium measuring 0.2–0.4 μm in width and 0.4–1.0 μm in length. Consistent with a bi-phasic developmental cycle, *C. burnetii* exists as two morphologically diverse cell types, namely an environmentally stable, non-replicating, extracellular small-cell variant (SCV) that transforms into a metabolically active, replicating large-cell variant (LCV) after invasion of host cells [9–11]. *C. burnetii* harbors several innate qualities that enhance this pathogen's potential use for biological warfare. This includes the potential for aerosol dissemination, a very low infectious dose, the significant morbidity caused by infection and the environmental stability and resistance of the SCV bacterium to eradication [11]. As such, *C. burnetii* has been classified by the US Centers for Disease Control and Prevention (CDC) as a Category B Bioterrorism Agent [12, 13] and Select Agent [14]. Additionally, the US National Institutes of Allergy and Infectious Diseases has categorized *C. burnetii* as a Category B Priority Pathogen [15].

Despite the inability of *C. burnetii* to cause enormous fatalities in the same manner as Category A Bioterrorism Agents such as *Bacillus anthracis* (causative agent of anthrax) and Ebola virus, the use of *C. burnetii* as a bioweapon is still significant due to the long-term consequences of persistence once released into the environment [16]. Whether *C. burnetii* has ever been deliberately used as a biological weapon is unclear; however, during the Cold War both the former Soviet Union and the USA weaponized *C. burnetii* in their offensive biological weapons programs [17]. Ken Alibek, the former deputy chief of the Soviet Union's biological warfare agency Biopreparat, has provided valuable insight into the full extent of the Soviet biological weapons program following his defection to the USA in 1992 [17]. Studies conducted by the US Army under the banner of Project Whitecoat at Fort Detrick, USA in 1955 effectively demonstrated the ease in which *C. burnetii* can be utilized as a biological weapon. The aim of Project Whitecoat was to ascertain the level of human vulnerability to attack from several pathogens [18]. Within this project, Operation CD-22 deliberately infected human volunteers with *C. burnetii* to investigate infectious dose, incubation period and symptom development. Exposure to aerosolized *C. burnetii* by conscientious objectors, namely members of the Seventh-day Adventist Church who hoped to serve their country without taking up

arms, revealed the minimum infectious dose as one to ten bacteria [19]. Furthermore, an aerosolized biological attack was simulated in Utah at the Dugway Proving Ground in which volunteers, alongside guinea pigs and primates, were exposed to *C. burnetii* that was released just over 900 meters away from test subjects [20]. In addition to the USA and former Soviet Union, the notorious Japanese cult Aum Shinrikyo, notably known for their dissemination of the deadly nerve agent sarin in the Tokyo subway system in 1995, also experimented with numerous biological warfare agents, including *C. burnetii* [21].

9.1.3 Culturing the Uncultivable: Significant Advances in *C. burnetii* Research

Historically, cultivation of *C. burnetii* involved repeated passage through either animal models such as guinea pigs or embryonated eggs. The inability to grow this bacterium in axenic media, resulted in the designation of *C. burnetii* as an obligate intracellular pathogen and significantly hampered research into the pathogenesis of this organism [22]. Nevertheless, it has long been known that the virulence potential of *C. burnetii* is associated with antigenic variation in lipopolysaccharide (LPS) expression. The infectious form, termed phase I, is characterized by the presence of smooth, full-length LPS and is isolated from natural sources including infected arthropods, animals, and humans [23]. The avirulent phase II form is obtained only after extensive serial passages in tissue culture cells or immunocompetent hosts including embryonated egg cells in the laboratory [24]. Phase II *C. burnetii* acquire a severely truncated, rough LPS that, despite containing core sugars and lipid A, is missing the *O*-antigen due to a large chromosomal deletion [25–27]. Genetic deletion leading to phase II transition is irreversible. This is of significant benefit to *Coxiella* researchers since avirulent phase II *C. burnetii* are suitable for use in biosafety level 2 laboratories in contrast to phase I variants that require biosafety level 3 containment [28]. The most widely used strain to study host-pathogen interactions is the phase II variant of the Nine Mile strain (NMII) since the growth dynamics and intracellular trafficking in human macrophages are indistinguishable from the phase I Nine Mile variant [29, 30].

The discovery that metabolic activation of *C. burnetii* required a moderately acidic pH of 4.5, analogous to a phagolysosome [31], and subsequent analyses of the metabolic requirements of *C. burnetii* using pathway analysis, metabolite typing, and transcriptomics resulted in the development of cell-free laboratory culture medium that could support the growth of *C. burnetii* [32, 33]. Substantial growth of *C. burnetii* was observed when cultivated in this acidified citrate cysteine medium (ACCM) with a pH 4.75, especially when incubated in a 2.5% oxygen atmosphere [33]. Subsequently, addition of methyl-β-cyclodextrin greatly improved growth of *C. burnetii* in both liquid form and as colonies embedded in semi-solid media [34]. The implications of this significant advance in the *Coxiella* field is two-fold. The ability to cultivate *C. burnetii* axenically has resulted in the development and application of genetic tools that have enhanced our identification and understanding

of virulence determinants essential for bacterial interaction with eukaryotic host cells [35–38]. However, establishment of laboratory culture medium, although compositionally quite complicated, may have also inadvertently made it easier for mass production of *C. burnetii* and consequently enhanced its potential for use in biological warfare and bioterrorism.

9.2 Taking the "Query" Out of Q Fever

9.2.1 Epidemiology of Human Infection

Q fever is found worldwide, with sporadic outbreaks described in many countries, although no cases have been reported in New Zealand or French Polynesia [22, 39]. Several seroprevalence studies have been conducted across Europe to determine disease incidence in either random population samples, blood donors, or at-risk individuals involved in handling livestock [40]. Seroprevalence differs widely among countries, with a study in the Netherlands noting almost 84% seroprevalence in at-risk groups such as veterinarians and those in close proximity to farms [41]. Nevertheless, these studies may not accurately reflect the true prevalence of *C. burnetii*, as each utilized different testing methods and antibody titer cut-offs to determine seropositivity [40].

The main reservoirs of *C. burnetii* are ruminants, such as cattle, goats, and sheep [42]. *C. burnetii* has also been detected in other animals, including other mammals, birds, and arthropods such as ticks [22]. The exact prevalence of *C. burnetii* in these animals are not clearly defined, since most studies only note seroprevalence and do not isolate bacteria [22]. A broad range of clinical signs have been observed in different animals. For instance, a systemic infection involving splenomegaly, hepatitis, and pneumonia can occur in laboratory mice and guinea pigs whereas infection of ruminants is generally subclinical [43]. Importantly, *C. burnetii* can cause abortion and stillbirth in pregnant mammals [44–46].

Q fever outbreaks occur across the world, including, in recent years, in the USA, Poland, and Australia [47–49]. However, the largest outbreak of Q fever occurred in the Netherlands between 2007 and 2010 [50, 51]. Over 4000 patients were confirmed to have been infected with an assumption that many more may have been affected but not properly diagnosed [52]. The outbreak was first reported by doctors in late May 2007, who noted an increased prevalence of pneumonia amongst adult patients [50]. Although initially misdiagnosed as *Mycoplasma pneumoniae* infections, further tests confirmed that the infections were indeed acute Q fever caused by *C. burnetii* [50]. A study traced the source of the outbreak to a small region in the Netherlands, which had a high density of livestock including cattle, sheep, and goats [53]. One of these goat farms experienced a wave of abortions in the livestock during April 2007 [53]. Several public health measures were implemented by the Dutch authorities to control the Q fever epidemic, including notification of abortion cases in herds, vaccination of animals within the affected areas, and later more drastic measures, including systematic culling of gestating ewes and goats

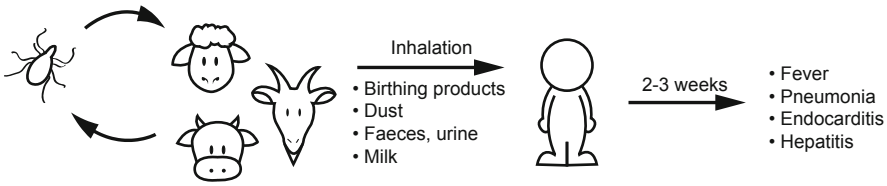

Fig. 9.1 Transmission of *Coxiella burnetii*. Ticks can harbor *C. burnetii* and transmit it to wildlife, especially sheep, cattle, and goats, which are the main reservoirs of human infection. *C. burnetii* is predominantly transmitted to humans through inhalation of contaminated particles from dust, birthing products, feces or milk, after which it resides in the lungs of individuals. Infected people either remain asymptomatic or disease outcomes such as febrile illness or pneumonia become apparent within 2–3 weeks. In severe cases, infection can lead to endocarditis or hepatitis

[52]. The outbreak was eventually contained with a cost in excess of 300 million Euro [54], and over 50,000 goats and sheep were culled between December 2009 to June 2010 alone [51]. This outbreak highlights the significant risk that a large Q fever outbreak could pose, evidenced by the heavy economic and public health burden faced by the Netherlands.

9.2.2 Transmission

As mentioned previously, *C. burnetii* commonly infects ruminants, and many Q fever outbreaks have traced the infection source to these animals [22]. The bacteria are shed from their hosts into the environment in milk, feces, urine, and also in particularly high numbers in reproductive products such as the placenta and the amniotic fluid, which are released into the environment during infection-induced abortions (Fig. 9.1) [22]. The bacterial titer in the placenta can reach 10^9/g in goats, indicating that abortion as a route of excretion is very important for transmission of *C. burnetii* [55]. The strong link between transmission and parturition means that outbreaks commonly occur during ruminant birthing seasons [56]. Once excreted, environmental *C. burnetii* is particularly hardy, resistant not only to UV radiation, but also osmotic pressure and desiccation [22]. This means that once *C. burnetii* contaminates a certain environment they are extremely difficult to eradicate and there is significant potential for these bacteria to cause multiple infections over time.

C. burnetii is transmitted to human hosts primarily through aerosol inhalation (Fig. 9.1) [57, 58], and upon inhalation, *C. burnetii* typically infects alveolar macrophages inside the lungs [29]. Aerosol based transmission of *C. burnetii* means that infection can occur even when patients have not been in contact with animals or animal products [53, 57]. Strong winds and dry, warm weather, are significant contributing factors for increased transmission [59]. This was the case during the Netherlands Q fever outbreak, where the weather preceding the beginning of the outbreak in 2007 was unusually warm and dry, with a predominant easterly wind present [50, 53]. Ingestion of contaminated products, for instance through the consumption of contaminated dairy produce, is also a possible mode of transmission

to humans in some outbreaks [60]. Animal-to-animal and animal-to-human transmission is common. However, human-to-human transmission of Q fever is extremely unlikely. Nevertheless, a study identified a person who contracted Q fever through exposure to human birthing products [61].

In rare instances, arthropods such as ticks may act as vectors of transmission for *C. burnetii* by feeding on infected vertebrates and ingesting the bacterium through the blood [22]. However, many studies have only looked at tick transmission during laboratory settings and no human outbreaks have been traced back to a tick-borne source [22, 62]. In fact, early studies linking *C. burnetii* carriage and transmission in ticks are now in doubt, as advances in molecular techniques and sequencing have shown that the bacteria in ticks were not *C. burnetii* [62].

9.2.3 Clinical Features

In humans, approximately 40% of infected individuals experience acute illness, with symptoms typically arising 2–3 weeks after infection [63]. Acute Q fever has a variety of clinical presentations and is often described as an influenza-like illness, with predominant clinical signs including fever, headaches, chills, myalgia, and fatigue [53, 64, 65]. Other acute Q fever presentations include hepatitis and atypical pneumonia, which may be visible via a chest X-ray even if the patient does not present with respiratory distress [64, 65]. Hepatitis during Q fever does not necessarily result in jaundice, but elevated hepatic enzyme concentrations are usually detected [22, 64]. These clinical signs of infection may not all occur in an individual at once, with various combinations observed throughout acute infection [53, 64]. Another important aspect of acute Q fever is infection during pregnancy. In the absence of appropriate treatment, acute Q fever in pregnant women has been associated with abortion, stillbirth, growth retardation, and low birth weight [66–68].

Curiously, particular clinical signs appear more prevalent in certain geographical locations. For instance, a study of 323 cases of acute Q fever in France showed hepatitis was seen in 61.9% of infections [64]. Hepatitis is also the most commonly reported clinical sign in Australian Q fever cases [69]. In contrast, during the largest outbreak of Q fever in the Netherlands, pneumonia was the most common sign and hepatitis was rarely seen [53, 70]. Whether these differences are due to infections by different strains is currently unknown since many outbreaks are not characterized for strain specificities. Genome sequencing may detect endemic strains in certain geographical locations, which may aid in tracking and understanding the pathogenicity and predominant clinical signs of distinct *C. burnetii* strains.

Acute Q fever can last from a couple of days to several weeks and in some instances the infection can become chronic [64, 71]. The mechanisms that lead to chronic infection are currently unknown, and progression does not appear to be strain-related [72]. However, these patients typically have underlying cardiac or vascular pathologies and other immune deficiencies. Increased age and pregnancy have also been implicated as factors which can increase the likelihood of developing chronic disease [72–75]. The most common clinical sign of chronic Q fever is

endocarditis, with symptoms appearing a few months to years after initial exposure [76, 77]. Notably, early reports of chronic Q fever following the Netherlands outbreak have shown that vascular infections were seen in 122 out of 284 chronic Q fever patients, whereas endocarditis was only present in 75 cases [74]. These clinical presentations, in combination with increased age, inadequate antibiotic intervention, and underlying cardiac or vascular pathologies, increase the lethality risk significantly [22, 74]. Chronic fatigue is also known to be a major long-term symptom for those with either acute or chronic Q fever [69, 78, 79]. One study found as many as 37% of Q fever survivors suffered from fatigue and general impaired health 2 years after the onset of acute illness [80]. The combined costs from the loss of income and increased sick leave because of chronic fatigue symptoms pose a significant economic burden in affected countries [54].

9.2.4 Diagnosis

Due to the clinical signs of acute Q fever being indistinguishable from influenza and other febrile illnesses, the US CDC recommends a serological test coupled with polymerase chain reaction (PCR) for a definitive diagnosis [81]. An indirect immunofluorescence assay is the most commonly utilized serological testing method for acute Q fever, particularly in the US [22, 81]. Seroconversion against *C. burnetii* occurs between 1 and 2 weeks after initial infection, with antibodies against phase II *C. burnetii* seen during the early stage of infection [82]. Generally as the infection progresses, phase I antibodies become more prevalent [22]. Samples taken during the initial acute phase and the convalescent phase of infection are compared and a four-fold increase in phase II antibody titers against *C. burnetii* antigens confirms the diagnosis [81]. A major setback for the serological testing method is that the tests can only be performed a few weeks after the initial onset of infection [83]. Therefore, PCR is highly useful for early detection of Q fever [81, 83, 84]. The most routinely utilized target for diagnosis is the IS1111 insertion element, although the sensitivity varies depending on the strain [85–87]. Serological testing is primarily used for the diagnosis of chronic Q fever, particularly by testing for the presence, or increases in antibody titers against phase I *C. burnetii* [81, 88]. More recently, following the Q fever outbreak in the Netherlands, a systematic review detailed a more defined diagnostic tool for chronic Q fever. This new guideline recommends a combination of PCR and serological tests against phase I antibodies, and a diagnosis for endocarditis or detection of visible infection via an imaging system [88].

9.2.5 Treatment

The standard and most effective treatment of both acute and chronic Q fever is doxycycline, with or without hydroxychloroquine [89, 90]. Since doxycycline treatment of pregnant women with Q fever is contraindicated, long-term co-trimoxazole therapy is the recommended alternative treatment regimen

[66]. For chronic Q fever, treatment lasts a minimum of 18 months, with antibiotics required to be taken twice-daily as per an acute infection during this time [89]. Side effects include photosensitivity of the hands and nose, irreversible cutaneous pigmentation, and retinal accumulation of hydroxychloroquine. These side effects, along with the difficulty of long term compliance during treatment, mean that an alternative and more effective method of treating chronic Q fever is necessary [89].

Currently the only commercially available human vaccine to protect against *C. burnetii* infection is a formalin-inactivated whole-cell vaccine containing the phase I Henzerling strain, called Q-Vax [91]. This vaccine is licensed for use in Australia, and is offered to at-risk individuals including abattoir workers and farmers [92]. Q-Vax is effective at preventing infection in those that are at high risk of exposure [93]; however, a two-step screening process is required before administration. This screening process involves identifying the presence of antibodies against Q fever, and conducting an intradermal skin test using diluted vaccine [93]. Screening for sensitization is an important process, as it was found that individuals who have pre-existing immunity can have adverse side effects to the vaccine [91, 94]. Other Q fever vaccines do exist, ranging from attenuated live cell, acellular extracted, and whole-cell vaccines using inactivated bacteria [91]. However, many of the studies examining these vaccines did not involve any control groups, making the true efficacy difficult to determine [91]. In recent years, livestock-specific vaccines have been under development with Coxevac, consisting of inactivated phase I Nine Mile *C. burnetii*, being the most effective at preventing shedding of bacteria in infected animals [95–97]. However, this vaccine is not available in all countries, with one study in Australia noting that the researchers were unable to obtain an import permit for this vaccine due to biosecurity concerns [48]. This study concluded that efforts will be made to develop a local vaccine for livestock, since Q-Vax is too costly for use in livestock [48].

9.3 Intracellular Life of *C. burnetii*: Characteristics of the *Coxiella*-Containing Vacuole (CCV)

9.3.1 Entry and Endocytic Traffic

C. burnetii appears to have a tropism for phagocytic cells, particularly alveolar macrophages, during human infection. However, this pathogen is able to invade and replicate inside many different cell types. *C. burnetii* passively enters phagocytic cells via actin-dependent phagocytosis [98]. This process is believed to involve interaction between the pathogen and $\alpha_v\beta_3$ integrin on the host cell surface [99]. Recently a *C. burnetii* outer membrane protein, OmpA was identified as an invasin that facilitates a zipper-like mechanism of invasion of non-professional phagocytes but the host receptor that interacts with OmpA is still unknown [100].

Once internalized, the vacuole containing *C. burnetii* is trafficked through the endocytic pathway via a series of ordered interactions with early endosomes, late endosomes, and lysosomes (Fig. 9.2). Unlike other intracellular pathogens, which

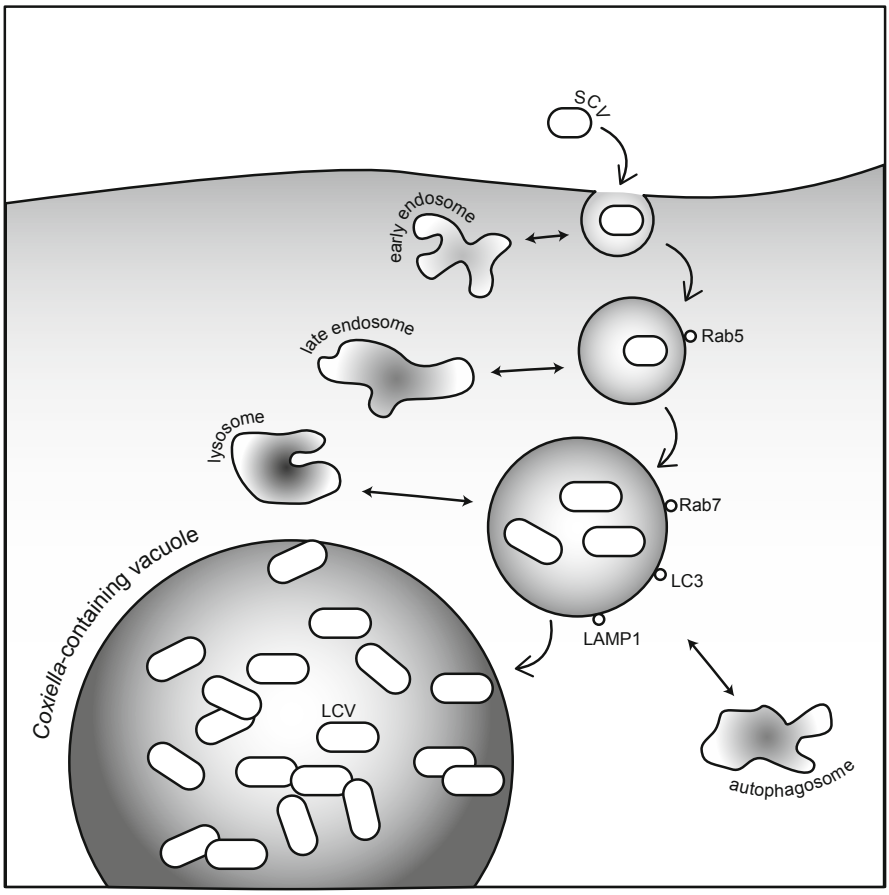

Fig. 9.2 Intracellular trafficking of *Coxiella burnetii*. *C. burnetii* resides in the environment as a stable small-cell variant (SCV) prior to entering a host cell through either bacterial-mediated means or passive phagocytosis. This *C. burnetii*-containing phagosome matures along the endocytic pathway interacting first with early endosomes, acquiring the marker Rab5. The maturing vacuole subsequently interacts with late endosomes and lysosomes, obtaining markers of these vesicles, such as Rab7 and LAMP1, and acquiring the characteristics of these vesicles, including low pH and hydrolytic activity. Exposure to these conditions induces the pathogen to transition into the replicative large-cell variant (LCV) and induces further changes to form the *Coxiella*-containing vacuole (CCV) that supports replication. The CCV is highly fusogenic, interacting with components of many vesicular trafficking pathways, and expands to occupy the majority of the host cell cytoplasm. Interaction with autophagosomes recruits LC3 to the lumen of the CCV

use virulence factors to either subvert endocytic maturation of the phagosome or escape the phagosome, to avoid the bacteriolytic confines of the lysosome, *C. burnetii* thrives within this hostile environment [101]. Early reports indicated that endocytic maturation of engulfed *C. burnetii* is delayed by the pathogen taking approximately 2 h after ingestion compared to 15 min for an inert particle to reach the lysosome [102]. This delay may be linked to interaction with autophagosomes

[103], but the advantage and the molecular mechanisms behind this slight stall in endocytic maturation are yet to be determined. The essentiality of delivering *C. burnetii* to the lysosome to initiate virulence means that disease progression is reliant on this vesicular trafficking pathway and the key regulators of endocytic traffic including Rab5 and Rab7 [104, 105]. Similarly, numerous host SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins are enriched on the CCV, including Vamp3, Vamp7, Vamp8, and Vti1b [106]. The SNARE family of proteins are responsible for docking and fusion events during vesicle-mediated transport (e.g., Vamp8 mediates fusion between late endosomes, and Vamp7, accompanied by Vti1B, aids heterotypic fusion of late endosomes with lysosomes) [107]. siRNA-mediated silencing of endogenous Vamp7 results in smaller CCVs, highlighting the importance of this host factor for bacterial growth [106].

Conditions within a host lysosome induce transition of *C. burnetii* SCVs to the metabolically active and replicative LCV form [10]. This transition also involves a shift to virulence and the pathogen directs modulation of the CCV to support bacterial replication [101]. *C. burnetii* will replicate in this lysosome-derived vacuole for approximately 6 days before LCVs eventually differentiate back to SCVs [10]. Throughout the intracellular replication cycle, the CCV maintains the low pH and hydrolytic characteristics of a lysosome. The mechanisms used by *C. burnetii* to survive these conditions are undefined. In comparison to other intracellular pathogens, the *C. burnetii* genome encodes mostly basic proteins with an average pI of 8.25. This feature is predicted to help the pathogen in buffering the low pH of the CCV [108]. Bioinformatic analysis has also identified four Na^+/K^+ exchangers and transporters for osmoprotectants that are predicted to help combat oxidative and osmotic stress within the CCV [108].

Despite maintaining the environmental traits of a lysosome, the CCV displays several intriguing characteristics that are directed by the pathogen. These include the rapid and dramatic expansion of the CCV, to eventually occupy most of the host cell's cytoplasmic space, and the strong induction of anti-apoptotic signaling within the host [101]. Additionally, recent reports have highlighted that *C. burnetii* utilizes host cholesterol metabolism and trafficking pathways to establish a successful infection. The CCV fuses with cholesterol-rich multivesicular bodies and both LDL-derived and endogenous cholesterol is transferred to the CCV [109, 110]. Interestingly, *C. burnetii* also recruits ORP1L (oxysterol binding protein related protein 1 long), a host cholesterol-binding protein to the expanding CCV [111]. Finally, CCV biogenesis and replication of *C. burnetii* is disturbed if host cell cholesterol metabolism is inhibited [112, 113].

9.3.2 Expansion of the CCV

Following delivery to the lysosome and LCV maturation, *C. burnetii* directs the expansion of the CCV even before the pathogen replicates. This expansion represents the fusion between CCVs and various types of cellular vesicles

[114]. This fusion includes endocytic vesicles, demonstrated by the coalescence of endocytosed material such as latex beads or other non-*Coxiella* microbes in the CCV, and homotypic fusion with other CCVs [115]. Most recently, several studies have demonstrated fusion of CCVs with autophagosomes [116–119]. These findings indicate that the mature CCV is most accurately described as maintaining an autolysosomal state of maturation [116]. The importance of autophagosome interaction with CCVs for *C. burnetii* virulence remains unclear. Early studies demonstrated that CCV development is dependent on autophagy leading to the idea that autophagosomes provide a nutrient source for the replicating *C. burnetii* [104, 120]. However, a more recent genome-wide siRNA study to identify host factors that contribute to CCV development indicated that silencing expression of central proteins in the autophagic process did not alter the CCV size or number [105]. It has also recently been observed that clathrin is enriched on the cytoplasmic face of the CCV membrane, leading to the suggestion that clathrin-mediated vesicular traffic is also diverted to the CCV contributing to the vacuole expansion [121, 122].

9.3.3 Anti-apoptotic Characteristics

Despite *C. burnetii* directing gross rearrangement of the host cell and the CCV growing to occupy the majority of the cellular space, the viability of infected cells is not impacted. Rather, infected cells become resistant to apoptotic stimuli in a manner that is dependent on bacterial protein synthesis [123, 124]. This is a crucial virulence trait as host cell viability must be maintained for an extended period to support the slow intracellular replication of *C. burnetii*. The action of both intrinsic and extrinsic stimuli of apoptosis are blocked during *C. burnetii* infection and this occurs through multiple mechanisms including blocking cytochrome c release from mitochondria, induction of pro-survival kinases such as Akt and cAMP-dependent protein kinase, and inducing a pro-survival transcriptional profile in the host cell [123, 124]. Both pro-apoptotic, for example Bad, and anti-apoptotic, for example Bcl-2, factors are recruited to the CCV membrane, which may alter its functional capacity and influence downstream signaling [125, 126].

9.4 Investigating the Pathogenesis of *C. burnetii*

9.4.1 Laboratory Models of Infection

Several laboratory animals, including laboratory mice, rats, guinea pigs, rabbits, and nonhuman primates, can be infected with *C. burnetii* resulting in various clinical presentations ranging from asymptomatic to febrile illness to death [22]. Generally, laboratory mice, guinea pigs, and nonhuman primates have been utilized to characterize the virulence of phase I *C. burnetii* infection [127–129]. Following initial isolation of *C. burnetii*, infection of guinea pigs rather than laboratory mice were

used since the disease progression in guinea pigs closely mimics human infection [130, 131]. Additionally, the non-human primate model, cynomolgus macaques (*Macaca fascicularis*) has been useful in the efficacy evaluation of several vaccines [132, 133]. A significant drawback of these models is that, as with humans, phase II *C. burnetii* is avirulent. This restricts the application of animal models to high containment laboratories and limits their application to investigate mutant libraries that have recently been generated with phase II *C. burnetii*.

To overcome these problems, alternative laboratory models have recently been proposed, including use of the greater wax moth (*Galleria mellonella*) larvae, which are susceptible to both phase I and phase II *C. burnetii* [134]. This study was the first to demonstrate in vivo characterization of *C. burnetii* NMII genetic mutants, creating an affordable, convenient model for researchers within the *Coxiella* field to determine the phenotypic impact of specific genetic mutations on *C. burnetii* [134]. There remain limitations to the usefulness of this model for investigating host-pathogen interactions during *C. burnetii* infection, including the unavailability of a complete genome of the greater wax moth, no methods for mutant generation and the inability of the greater wax moth to appropriately mimic the innate immune response in humans [135]. To overcome these limitations, the severe combined immunodeficient (SCID) mouse model, which possesses a normal innate immune system, has been successfully utilized to demonstrate the importance of several virulence factors of *C. burnetii* [136, 137]. This laboratory mouse model has the potential to characterize mutants that are attenuated for virulence in vivo, despite displaying no intracellular growth defects in vitro [136]. Despite these recent advances, the most common laboratory model for investigating *C. burnetii* infections are in vitro cell lines. Many cell lines support the replication of *C. burnetii*, including fibroblasts such as L929, epithelial cells such as HeLa and Vero, and murine macrophage-like cells lines such as J774 and P388D1 [31, 98, 138–142].

9.4.2 Hunting for Bacterial Virulence Factors

Development of axenic culture medium, ACCM, which allows the growth of *C. burnetii* in both liquid and solid culture, marked the beginning of a new dawn in *C. burnetii* research [32–34]. No longer relying on host cells for growth of the organism, traditional genetic manipulation approaches could be applied to *C. burnetii*. It is now possible to perform random mutagenesis using transposon systems, use reporter expression plasmids, and perform site-specific chromosomal gene knock-ins using a Tn7 system and targeted gene inactivation [35, 101, 143].

Taking advantage of these developments, two independent random mutagenesis studies by Martinez et al. [100] and Newton et al. [118] utilized the *Himar1* transposase system to identify virulence factors of *C. burnetii* that are necessary for establishing a successful infection within tissue culture cells. In the study by Martinez et al. [100], 1082 mutants were sequenced, annotated, and screened for their ability to infect and replicate within host cells and/or protect host cells from cell death. Similarly, Newton et al. [118] screened a library of over 3200 transposon

mutants for intracellular growth defects. Both studies validated earlier reports that the type IVB Dot/Icm secretion system is essential for intracellular replication of *C. burnetii* [36, 37]. Additionally, these screens identified the PmrAB two-component regulatory system as essential for intracellular replication and revealed several Dot/Icm substrates that play key roles in CCV biogenesis [100, 118]. Martinez et al. [100] were also able to identify and characterize OmpA as an outer membrane invasin that facilitates *C. burnetii* entry into non-phagocytic cells.

Another genetic tool that was aided by ACCM development was the generation of reporter expression plasmids. The discovery that *C. burnetii* supports independent replication of vectors derived from the IncQ plasmid RSF1010 led to the creation of two reporter plasmids, pCBTEM and pJB-CAT-BlaM, enabling the identification of substrates that are secreted into the host cell by the translocation system of *C. burnetii* [144, 145]. Potential substrates are transcriptionally fused to β-lactamase (BlaM) and translocation into the host cell is detected using a cell-permeable fluorescent dye that changes from a green to blue signal if the β-lactam ring is cleaved by BlaM [146–148]. Moreover, other reporter plasmids have been generated by modifying the backbone of pJB-CAT to contain N- or C-terminal epitope tags including 3xFLAG or 2xHA facilitating the study of intracellular trafficking and potential binding partners of *C. burnetii* proteins [35]. Finally, the ability to assign specific phenotypic characteristics to individual genes requires an efficient method for targeted gene inactivation. Two methods have been applied to *C. burnetii*, the first using Cre-*lox*-mediated recombination and the second a loop-in/loop-out approach, with the latter method having been successfully employed by several groups [149–151].

Since the development of ACCM has allowed rapid advancement and expansion of the *Coxiella* genetic toolbox, our knowledge of the mechanisms of pathogenesis used by *C. burnetii* to cause disease and the specific role of certain proteins during this process has accelerated enormously.

9.4.3 The Dot/Icm Type IVB Secretion System: An Essential Virulence Factor

The first complete genome sequence of *C. burnetii* provided important insight into the evolution and pathogenesis of this organism, especially the revelation of a type IVB secretion system (T4BSS) highly analogous to the Dot/Icm T4BSS of *Legionella pneumophila* [108]. The T4BSS is a specialized apparatus, ancestrally related to conjugation systems that translocate bacterial proteins, termed effectors, into the host cell [152]. The *C. burnetii* genome encodes for all homologues of the 25 *dot/icm* genes in *L. pneumophila* of which several are functionally equivalent demonstrated by the ability of *C. burnetii* genes to complement the corresponding *L. pneumophila* mutants [108, 153–155]. As with *L. pneumophila* [156, 157], the *C. burnetii* Dot/Icm T4BSS is required for virulence, since mutants in structural components of this translocation apparatus result in bacterial strains incapable of

intracellular replication [36, 37, 149]. These findings were reinforced with the aforementioned studies by Martinez et al. [100] and Newton et al. [118], both of which observed complete intracellular growth defects in many independent *dot/icm* transposon mutants.

Despite the absolute dependence of both *L. pneumophila* and *C. burnetii* on the Dot/Icm T4BSS for virulence, there are significant differences in the activation of effector translocation activity. In contrast to *L. pneumophila*, in which the pre-assembled apparatus delivers effectors upon host cell contact [158], the T4BSS of *C. burnetii* is not active until the bacterium has trafficked to the acidified lysosomal compartment [159]. Indeed, silencing key components of the host endocytic pathway, including proteins that control membrane fusion with early and late endosomes such as Rab5 and Rab7, respectively, perturbs effector translocation by *C. burnetii* [148, 159]. In a similar manner to *L. pneumophila* [160], the PmrAB two-component system of *C. burnetii* is responsible for regulating the expression of the Dot/Icm T4BSS and many effector proteins [161]. Importantly, three independent studies demonstrated the significance of the two-component system to intracellular replication [100, 118, 161].

9.4.4 Identifying Substrates of the Type IV Secretion System

The essentiality of the Dot/Icm T4BSS for *C. burnetii* virulence is dictated by the large repertoire of effector proteins that are introduced into the host cell via this apparatus. Collectively, these proteins are responsible for manipulating host pathways and establishing the unique CCV replicative niche. To date, over 140 *C. burnetii* effectors of the Dot/Icm T4BSS have been identified [162]. A range of screening and bioinformatic approaches, including the presence of a Dot/Icm C-terminal translocation signal, eukaryotic-like domains, sequence similarity to known substrates, and existence of a PmrA regulatory motif upstream of the candidate effector, have been used to identify these effectors [37, 38, 144, 161, 163]. Validation of candidate Dot/Icm effectors must occur experimentally using translocation assays such as the aforementioned BlaM assay, and indeed many were originally empirically confirmed as effectors using the surrogate host *L. pneumophila* prior to the availability of genetic tools for *C. burnetii* [144, 162, 164, 165].

9.4.5 Elucidating Effector Function

The critical importance of numerous effectors for intracellular growth and CCV biogenesis has been highlighted in several mutagenesis studies [38, 100, 118]. Ongoing research to elucidate the function of these effectors and how they support *C. burnetii* virulence will reveal key information about the molecular pathogenesis of *C. burnetii* infection. Already it has been established through mutant studies and ectopic expression of individual effectors that specific effector proteins influence

host pathways including vesicular trafficking, lipid metabolism, host gene expression, autophagy and apoptosis.

Given the highly fusogenic nature of the CCV, *C. burnetii* effectors are likely involved in modulating host vesicular trafficking pathways [166]. Indeed, two effectors, CvpA and Cig57, have been shown to interfere with clathrin-mediated vesicular trafficking, using independent approaches to recruit clathrin to the expanding CCV [121, 122]. The effector CvpA uses several endocytic binding motifs to bind the clathrin adapter complex AP2 [121] and Cig57 requires only a single endocytic sorting motif to bind FCHO2, an accessory protein of clathrin-coated pits [122]. Mutants of either CvpA or Cig57 result in severe intracellular growth defects indicating that they are not functionally redundant and are required for CCV biogenesis. Additionally, silencing expression of clathrin, AP2 or FCHO2 also causes a replication defect, highlighting the importance of clathrin-mediated vesicular trafficking to biogenesis of the CCV [118, 121, 122].

In addition to CvpA and Cig57, the effector protein Cig2 (also known as CvpB) modulates host membrane transport. Screening of transposon mutants for replication defects revealed the importance of Cig2 for the homotypic fusion of CCVs [118, 167]. The *cig2* mutant resulted in a multi-vacuolar phenotype characterized by CCVs of one cell not fusing. Additionally, in the absence of Cig2, CCVs are defective in the normal recruitment of LC3, an autophagy protein, to the CCV. This multi-vacuolar phenotype was similarly observed when key autophagy regulators such as ATG5, ATG12 or syntaxin 17 were silenced, indicating a functional link between Cig2 and autophagy [105, 118]. Recent findings indicate that Cig2 can influence PI(3)P metabolism to promote fusion of autophagosomes and CCVs [117]. Furthermore, it appears that maintenance of CCV fusion with autophagosomes by Cig2 decreases the host tolerance to infection [116, 117]. Several other effectors may also influence the host immune response to *C. burnetii* infection. For example, IcaA inhibits the caspase-11-mediated non-canonical activation of the NLRP3 inflammasome presumably aiding host survival and *C. burnetii* replication [150]. Additionally, interaction with the mitogen-activated protein kinase (MAPK) pathway by effectors Cem9, CetCb4 and CetCb2 may also serve *C. burnetii* infection through alterations to host signaling pathways [168].

The intracellular replication cycle of *C. burnetii* within host cells is quite prolonged, lasting over a week, compelling the bacteria to find mechanisms that inhibit host cell death or apoptosis [30]. Anti-apoptotic activity occurs during *C. burnetii* infection, a process that is dependent on the T4BSS [36]. Three Dot/Icm effectors, AnkG, CaeA, and CaeB, block apoptosis [169, 170]. AnkG blocks intrinsic apoptosis when expressed ectopically and was shown to be potent at physiological levels by demonstrating survival of mouse dendritic cells infected with *L. pneumophila* expressing AnkG [164, 171]. The anti-apoptotic activity of AnkG depends on binding the host mitochondrial protein p32 and subsequent importin-α1-dependent translocation of AnkG to the nucleus [171–173]. CaeA localizes to the nucleus and inhibits both intrinsic and extrinsic apoptosis [174]. Finally, CaeB appears to interfere with ER homeostasis and induces robust protection against intrinsic apoptosis at the mitochondria level [170, 175]. Future

mutagenesis studies will delineate the relative contribution of these anti-apoptotic effectors to *C. burnetii* virulence although it is likely that additional, as yet unidentified, effectors also contribute to maintaining host cell viability throughout infection.

9.5 Perspective: *Coxiella burnetii*—A Cause for Concern?

As a Category B Bioterrorism Agent, *C. burnetii* is not considered a catastrophic threat to society as it does not have the potential to cause significant community lethality. However, the recent outbreak of Q fever in the Netherlands has demonstrated the devastating health and economic impact of widespread exposure to *C. burnetii*. This natural outbreak was the perfect storm of events with delays in communication between the agricultural and health sectors and the precise environmental conditions to support *C. burnetii* dissemination through both animal and human populations. The impact of this outbreak has highlighted that weaponization of *C. burnetii* would be a significant event with very serious consequences. However, this outbreak has also acted as a training ground for agricultural and public health departments to develop strategies to control *C. burnetii* spread. Of continuing concern is the challenge to eradicate *C. burnetii* from contaminated environments and the severe consequences of chronic human infections. Both of these factors contribute to the debilitating long term impact that a deliberate release of *C. burnetii* would have.

Recent advances in axenic cultivation of *C. burnetii* have paved the way for a slew of important studies to elucidate the pathogenesis of *C. burnetii.* These studies, deciphering the unique host-pathogen interactions directed by *C. burnetii*, not only inform our understanding of this pathogen but are contributing to our understanding of eukaryotic cell biology and immune defense more broadly. Functional characterization of the cohort of unique Dot/Icm effectors that mediate *C. burnetii* virulence has already yielded significant contributions to the broader field of cellular microbiology. Such research will aid our preparedness for future Q fever outbreaks caused by deliberate release or natural circumstances. This research will also inform the development of improved vaccination strategies and may lead to development of novel therapeutic and prophylactic approaches in the event of an outbreak.

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Francisella tularensis: Causative Agent of Tularemia and Biothreat Agent

10

Monique Barel and Alain Charbit

10.1 Introduction

The bacterium *Francisella tularensis* subspecies (ssp.) *tularensis* (also designated *F. tularensis* type A) is considered as a potential biological warfare agent and therefore is classified as Class A agent by CDC. Indeed, *F. tularensis*, one of the most infectious and virulent bacteria, is responsible for tularemia, a zoonosis that may be transmitted to humans through animal bites or contact with animals, contaminated body fluids or feces, or by consumption of foods of animal origin, particularly meat and milk products. Inhalation of a low dose of *F. tularensis* type A is sufficient to cause human disease with a case-fatality rate of 30–60% if untreated [1]. Thus, use of *F. tularensis* in aerosol attacks is a plausible scenario as is deliberate contamination of food or water supply systems. Estimation by CDC of the economic cost of a bioterrorist attack by aerosolized *F. tularensis* type A is close to 5.4 billion US dollars per 1,00,000 exposed people. In 2007, Eisen emphasized the need for further studies on natural *F. tularensis* transmission cycles, infection routes, and epidemiology [2]. The broad adaptation capacity of *F. tularensis* to extreme environments is a new challenge for scientists trying to understand the exact mechanisms of the host response to infection with this pathogen.

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10.2 Natural Transmission Cycles and Routes of Infection

The natural reservoirs of *F. tularensis* are diverse, with its natural cycle involving animals, and biting arthropods. Mammals, birds, amphibians, and invertebrates of more than 250 species have been reported susceptible to *F. tularensis* infection (Fig. 10.1). The most easily infected animals are rodents and lagomorphs, which can become severely ill. The environment is therefore frequently contaminated with *F. tularensis*-containing feces from infected rodents. Birds, sheep, cattle, and dogs are more resistant to illness. Carnivores are very susceptible to infection but rarely develop clinical disease, unless infected with a very high dose. Blood-sucking arthropods play a role as vectors and are an inexhaustible reservoir of the bacterium. Bacterial survival depends on temperature. It may survive below 0 °C for several months but above 10 °C for only a few days. The observed persistence of the bacterium in aquatic environments is probably due to its survival in aquatic protozoa, such as amoebae [3].

The cutaneous route is the most common penetration pathway for *F. tularensis*. Typically, infections occur after direct contact with infected live or dead animals, through handling of contaminated objects, or after a bite from blood-sucking arthropods. Infections can also occur through the conjunctival or mucosal (including gastro-intestinal) routes after exposure to contaminated liquids or skin or consumption of contaminated water or undercooked meat from an infected animal. Finally, manipulation of animals or contaminated products and rural work or gardening may

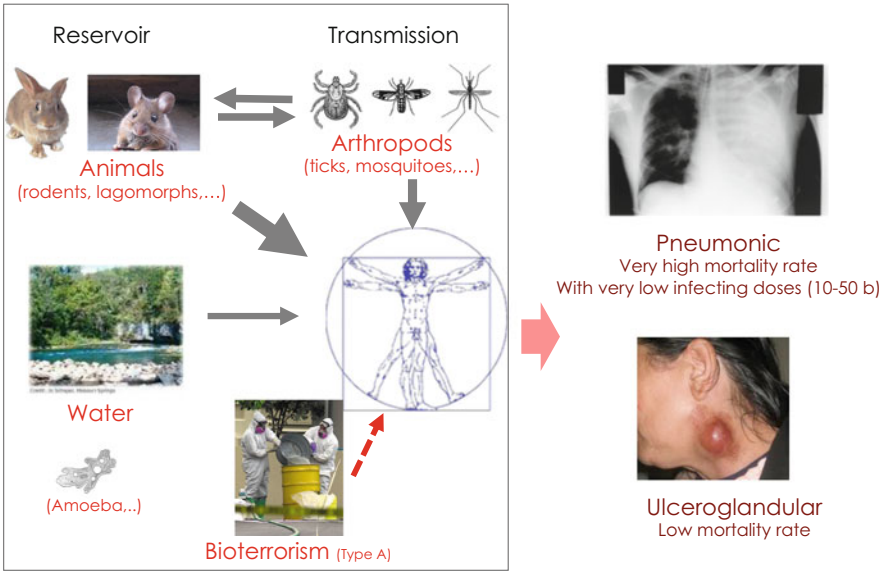

Fig. 10.1 Natural lifecycle of *F. tularensis*, involving animals, biting arthropods, and the environment. Description of two *F. tularensis*-associated pathologies associated with either the lowest or the highest case fatality rate

result in aerosolization of contaminated materials and lead to pulmonary infection. This route of infection causes the most severe form of *F. tularensis* infection, pneumonic tularemia. However, no inter-human transmission has been described and consequently isolation of tularemia patients is not necessary. 5–10 bacterial cells suffice to cause pneumonic tularemia, which has a high case fatality rate (30%), but 10^6–10^8 bacterial cells are required for gastrointestinal infection or to cause ulceroglandular tularemia, which has a low case fatality rate.

10.3 Epidemiology, Detection, and Treatment

The most virulent *F. tularensis* subspecies are *tularensis* (or type A) and *holarctica* (also designated type B or *palearctica*). Type A is mainly found in the forests of Northern America, in the US, whereas type B is present in continental Europe, as well as in Russia and Japan. New strains were also isolated from seawater in southern China [4] and in Tibet [5]. Subspecies *mediasiatica* is found in central Asia, and subspecies *novicida* is distributed in Northern America and Australia. Cases of tularemia peak in winter, corresponding to hunting season, and in summer, which is the period of maximal ticks and mosquito activity.

In recent decades, the number of tularemia cases remarkably increased in the Balkan countries compared to the incidence of tularemia globally. After the war in Kosovo (February 1998–June 1999), an outbreak occurred, with the number of human cases reaching 1221 between 1999 and 2010. This number reflects the highest recorded annual incidence of tularemia in Europe, corresponding to a rate of 5.2 per 1,00,000 people. Sweden, Finland, Slovakia, Czech Republic, Norway, Serbia, Montenegro, Hungary, Bulgaria, and Croatia follow with rates of 2.80, 1.19, 1.0, 0.81, 0.42, 0.4, 0.36, 0.21, and 0.15 per 1,00,000 people, respectively [6]. A recent report also described the re-emergence of tularemia in humans in Iran and its neighbors countries [7]. Thus far, tularemia has not been reported in UK, Iceland, Africa, South America or Antarctica. Of note, two cases of tularemia in ringtail possums, caused by *F. tularensis* subspecies *holarctica*, were very recently reported in Australia [8], suggesting that these animals could constitute a previously underestimated natural reservoir for this pathogen.

In Europe *F. tularensis* transmission uses two main natural reservoirs, either terrestrial by direct contact with animals or aquatic through contaminated water [9]. These two main natural reservoirs may be at the origin of the geographical distribution of the reported tularemia cases. Furthermore, a recent paper [10], which used a reverse genomic approach, also demonstrated, through variable replication events and long-range geographical events, that *F. tularensis* had moved in Europe from East to West. Altogether, these parameters are likely to account for the genetic diversity found among *F. tularensis* isolates.

In 2007, the WHO formulated a guideline, for distinguishing between suspected, presumptive and confirmed tularemia cases. However, one should keep in mind that these distinctions are mainly supported by the availability of detection tests in the

different affected countries. For example, the cases of human tularemia described in Iran, described as reemerging disease [7], are most likely due to the lack of available and efficient tests. Even in the USA, doctors were puzzled by the apparent surge of tularemia cases in 2015 in four US states (*i.e.* Colorado, Nebraska, South Dakota and Wyoming). Indeed, while about 125 annual cases of the disease had been referred over the past two decades in the whole country, 100 cases were reported by the CDC during the first 9 months of 2015 in these four states (43 cases in Colorado, 21 in Nebraska, 20 in South Dakota, and 16 in Wyoming). These 100 cases involved people aged from 10 months to 89 years. Because of this largely asymptomatic infection, one 85-year-old man died from the disease and 48 others had to be hospitalized. Until 2005, the only test kits available for diagnosis of human tularemia were serological in nature [11]. Since 2010 [12], technological advancements enabled the establishment of a wide array of new detection methodologies for *F. tularensis* (Table 10.1), some of them enabling on-site detection (Point-of-Care). Better estimation of the true prevalence could be obtained by sero-epidemiological surveys [9].

Treatment of diagnosed tularemia is mainly antibiotherapy. A thorough study on antibiotic susceptibility of *F. tularensis* strains was recently reported [23].

Each case of tularemia is treated according to its form and severity. Early diagnosis allows for immediate treatment with antibiotics. Several classes of antibiotics have been recommended for tularemia treatment, but their efficacy varies. Antibiotics that may be used to treat tularemia include: streptomycin, gentamicin, ciprofloxacin and tetracyclins.

Streptomycin is the drug of choice based on experience, efficacy and FDA approval. It is usually used to treat oculoglandular tularemia. The drug is given intramuscularly, twice a day, for 1–2 weeks. Surgical intervention may be required to drain swollen lymph nodes or to cut away infected tissue from a skin ulcer. Gentamicin is considered an acceptable alternative, but some series have reported a lower primary success rate. Treatment with these aminoglycosides should be continued for 10 days. Depending on the type of tularemia being treated, doctors may also prescribe instead oral antibiotics such as doxycycline (Oracea, Vibramycin) or tetracycline (Sumycin). They may work but are less effective than streptomycin. Ciprofloxacin and other fluoroquinolones are not FDA-approved for treatment of tularemia but have shown good efficacy in vitro, in animals, and in humans. However, one outbreak in Spain with 142 patients showed good clinical outcomes with ciprofloxacin; fluoroquinolone antibiotics may be useful in treating tularemia if additional studies will show clinical effectiveness. Tetracyclines may be a suitable alternative to aminoglycosides for patients who are less severely ill. Tetracyclines are static agents and should be given for at least 14 days to avoid relapse. Pneumonic tularemia is the form of the disease, which is associated with the highest case fatality rate compared to other *F. tularensis* infections. Therefore, development of new antibiotic therapies focuses primarily on this form, as it is also the most likely route of infection during a bioterrorist attack. Delivery of an antibiotic formulation via the inhalation route may potentially provide high antibiotic concentration at the site of infection (respiratory track) with reduced systemic

Table 10.1 Technology used for detection of *F. tularensis* since 2014

Technology used	Samples	Limit of detection	Criteria	Usable for detection	References
Biobarcode assay with on-chip capillary electrophoresis	Multiplex biological medium	50 CFU/mL	Rapid, sensitive, multiplex and accurate	Laboratory workers	[13]
Solid Phase recombinase polymeras amplification	144-bp double stranded DNA	6105 copies in 50 μL		Laboratory workers	[14, 15]
Lateral Flow assays	Bacteria		Limited sensitivity	Laboratory workers	[16]
Lateral Flow assays	Bacteria and complex samples	100 CFU/ test	Rapid (15 min) and sensitive	Point-of-care testing for first-level emergency response	[17]
Immunofiltration assays	Bacteria		High sensitivity but limited sensitivity and complicated for First responders	Laboratory workers	[16]
DNA microarray technology	Microarray probes		More facility and flexibility for designing professional microarray probes	Laboratory workers	[18]
Proteome microarrays	1741 different proteins derived from strain Schu S4 and sera			Diagnosis in a deployable format, the immunostrip	[19]
Magnetic beads with multiple capturing antibodies and genetically-engineered apoferritin protein constructs conjugated with quantum dots	Antibodies	10-fold increase in sensitivity compared to enzyme-based detection methods		On-site detection	[20]

(continued)

Table 10.1 (continued)

Technology used	Samples	Limit of detection	Criteria	Usable for detection	References
Latex agglutination test	Serum		Specific, sensitive, fast, easy-to-perform and cost-efficient tool	Routine diagnosis	[21]
GeneXpert system	Whole blood	10 CFU/mL	Highly sensitive	Point-of-care detection	[22]

exposure. Two liposomal ciprofloxacin formulations (Lipoquin® and Pulmaquin®) [24] are currently in development. These formulations could improve many parameters such as ciprofloxacin tolerability or dose frequency, and increased penetration of biofilms. Indeed, biofilms formed by multicellular communities of bacteria are known to constitute an environment potentially susceptible to increase antibiotic resistance and transmission of *F. tularensis.*

Immunotherapy is an alternative treatment path that is being explored due: (1) to the fear that weaponization of *F. tularensis* may include the creation of antibiotic-resistant strains [25], and (2) to the existence of natural strains resistant to erythromycin in Europe. Antimicrobial peptides (also known as host defense peptides or HDP) are being explored too. Cationic HDP have been shown to play a crucial role in the innate host defense system. The antimicrobial activity of cationic HDPs such as cathelicidins and defensins against *F. tularensis* is promising [26].

An efficacious, safe, and licensed vaccine against *F. tularensis* is still not yet available [27]. However, an attenuated live vaccine strain (LVS) derived from *F. tularensis* type B is available but only for protection of laboratory workers and other at-risk groups.

10.4 Intracellular Lifecycle

Living within a host cell allows a pathogen to evade acquired immunity. Indeed, once inside the mammalian cell, the pathogen is no longer susceptible to complement or neutralizing antibodies. *F. tularensis* is one among few bacteria that has evolved to live within professional phagocytic cells, such as macrophages. *F. tularensis* enters its host cell through receptor-mediated phagocytosis and traffics to the phagosome, from which it escapes to replicate in the cytosol [28]. The acidification status of the *F. tularensis*-containing phagosome (FCP) depends on the infection conditions [29]. Indeed, acidified FCPs are observed after using

non-opsonic infection conditions, whereas non-acidified FCPs are generated when serum-opsonized *F. tularensis* enters the cell. Intra-cytoplasmic multiplication is accompanied by degradation of cellular glycoproteins, the by-products of which may serve as nutritional substrates for the bacterium [30].

In amoebae, *F. tularensis* resides and replicates within non-acidified, membrane-bound vacuoles, within the trophozoites [31]. In contrast, in mammalian and arthropod cells, phagosomal escape of *F. tularensis* is dependent on the *F. tularensis* pathogenicity island (FPI) genes, which encode a Type VI Secretion System (T6SS), and the unfoldase ClpB [32]. *F. tularensis* may also invade mammalian erythrocytes in the absence of phagocytosis or endocytosis [33] and the T6SS is required for erythrocyte invasion. Recent evidence indicated that residing within a mammalian erythrocyte enhanced the ability of *F. tularensis* to colonize ticks following a blood meal on the mammal. Erythrocyte residence also protects *F. tularensis* from a low pH environment, a similar low-acidic environment as to that of gut cells of a feeding tick [33]. A rapid escape of *F. tularensis* from the hostile environment of the phagosomal compartment with its degradative enzymes and reactive oxygen species (ROS is critical for its pathogenicity). We recently showed that a glutamate transporter of *F. tularensis* (GadC) was critical for oxidative stress defense in the murine phagosome, thus impairing intra-macrophage multiplication and virulence in a mouse model of tularemia [34]. Links between glutamate uptake, oxidative stress defense, the tricarboxylic acid cycle, and phagosomal escape, were highlighted. Depending on the virulence of the *F. tularensis* strain and its sensitivity to ROS [35], activation of the AIM2 inflammasome may be triggered. Inflammasomes are cytosolic cellular sensors of harmful situations such as the presence of microbes. Upon activation, inflammasomes trigger the proteolytic maturation and release of inflammatory cytokines then initiating immune and repair responses. Assembly of inflammasomes relies on a diverse repertoire of sensor proteins that can detect specific stimuli. For example, the AIM2 inflammasome is activated by the presence of microbial DNA within the cytosol. Although virulent *F. tularensis* type A does not trigger activation of the host AIM2 inflammasome, non-pathogenic *F. novicida* do trigger delayed AIM2 inflammasome and this process in dependent on mitochondrial ROS.

The intracellular niche protects F. *tularensis* against competing bacteria and provides a unique source of nutrients. Indeed, adaptation to the nutrient-rich intracellular environment may have led to *F. tularensis* genome reduction [36], by purging unnecessary metabolic genes. Among the nutrients required to support *F. tularensis* replication, iron is essential for key enzymatic and redox reactions [37]. The *F. tularensis* genome encodes proteins for two distinct pathways that allow iron acquisition: a siderophore-dependent ferric iron uptake system and a ferrous iron transport system. Amino acid transporters are also major players in the adaptation of intracellular pathogens [38]. An asparagine transporter required specifically for cytosolic multiplication of *F. tularensis* [39] and a new member of the major super family (MSF) of transporters involved in isoleucine uptake are also encoded by *F. tularensis* [40]. These transporters play critical roles in intracellular metabolic

adaptation of *F. tularensis*, which uses host amino acid as major gluconeogenic substrates [41].

10.5 Host Immune Response

Technological advances, such as "Multi-Omic" approaches, allow host-pathogen interactions to be studied in much greater detail. A better understanding of the mechanisms involved should facilitate the development and refinement of new and existing vaccines and therapeutics. As an example, advances in bio-imaging provide noninvasive means for identifying the internal systemic spread of infection in animal models and the impact of a prophylaxis or a therapy on the disease process [42]. Gene signatures have been determined in the different organs of laboratory mice infected by *F. tularensis* using a DNA microarray. Using dual RNA-Seq, a marked suppression of multiple components of the murine innate immune response was found after an acute 4-h infection by *F. tularensis*. Furthermore, *F. tularensis* increased only a subset of immune-related transcripts in the mouse. However, a subsequent activation of the classical inflammatory response 48 h after exposure to the bacteria, was associated with altered abundance of *Francisella*-specific transcripts, including those associated with bacterial surface components [43].

Host response pathways modulated by *F. tularensis* infection include Toll-like receptor 2 (TLR2), caspase-1 inflammasome, interferon type I, NADPH oxidase, phosphatidylinositide 3-kinase (PI3K) and Ras pathways. For example, proinflammatory cytokines such as tumor necrosis factor alpha are not produced in infected murine cells [44]. Depending on the *F. tularensis* subspecies, inflammasome activation may also be delayed. *F. tularensis* mutants unable to escape the phagosome survive poorly in the cytoplasm and increase the expression of IFN-β and are cytotoxic. These mutants are attenuated in the tularemia mouse model, indicating that intracellular replication is necessary for *F. tularensis* virulence. A type II IFN activation of the inflammasome has been observed *in vivo* as a compensatory mechanism in the absence of type I IFN response [45].

Among its multiple survival strategies, *F. tularensis* attenuates expression of an atypical, poorly inflammatory LPS [46]. Apart from LPS, which contributes to the stealth intracellular multiplication and high infectivity of *F. tularensis*, more "classical" virulence factors that interact with the hosts have been described recently [47], including outermost capsular layer and the outer membrane proteins of the bacterial cell.

10.6 Host Metabolic Response

Metabolic reprogramming of the infected host cells constitutes a key component of *F. tularensis* intracellular parasitism. It accounts for both the inhibition of host defense mechanisms and the optimal bacterial replication. Oxidative phosphorylation shifting to aerobic glycolysis is a requirement in macrophage metabolism for

actively combating pathogen invasion. *F. tularensis* capsule impairs this shift by preventing production of inflammatory cytokines [48]. *F. tularensis* cytosolic growth is also enabled by manipulation of the autophagic pathway. Autophagy allows the orderly degradation and recycling of cellular components. Therefore, autophagy provides amino acids necessary for cytoplasmic growth of *F. tularensis.* In disease, autophagy has been seen as an adaptive response to stress, which promotes survival, whereas in other cases, it appears to promote cell death and morbidity. In the extreme case of starvation, the breakdown of cellular components promotes cellular survival by maintaining cellular energy levels. An additional function of autophagy, xenophagy, is also avoided through capsular and lipopolysaccharide *O*-antigen of *Francisella* [49]. Bacteria survival and multiplication in the cytoplasm may be also permitted by *O*-glycosylation increase of glycoproteins, which induce the down-regulation of the unfolded protein response (UPR) [50]. Expression of SHIP, a critical modulator of host resistance to bacteria, is down-regulated in monocytes and macrophages infected by *F. tularensis* subspecies *novicida.* SHIP is the known target of the microRNA miR-155. Cells infected by the virulent *F. tularensis* type A strain SCHU S4 led to a lower miR-155 response than the cells infected with *F. tularensis* subspecies *novicida.* This impaired miR-155 induction by SCHU S4 may account for the virulence of Type A *Francisella* [51]. Apoptosis is another pathway leading to macrophage death. It is induced by most of the FPI components, which are either secreted by the T6SS or belong to its core components. However, *F. tularensis* mutants, which do not sustain intracellular proliferation, are unable to induce apoptosis, suggesting that intracellular proliferation is necessary to trigger apoptosis [52].

10.7 Conclusion

Somewhat counter intuitively, the number of publications on tularemia listed in PubMed since 1946 are inversely proportional to the number of cases reported by the US CDC (Fig. 10.2). This observation suggests that improved knowledge of the clinical and biological aspects of tularemia has led to a better control of the disease. However, there is still risk of a tularemia epidemic. Indeed, the constant movement of humans across the globe and climate change-induced alterations of the environment with concomitant animal (arthropod, rodent, bird) migrations complicate the control of *F. tularensis* spread. In addition, the possibility of emerging antibiotic-resistant *F. tularensis* strains should not be neglected. Consequently, the development of improved diagnostic tests and prophylactic and post-exposure vaccines remains a high priority.

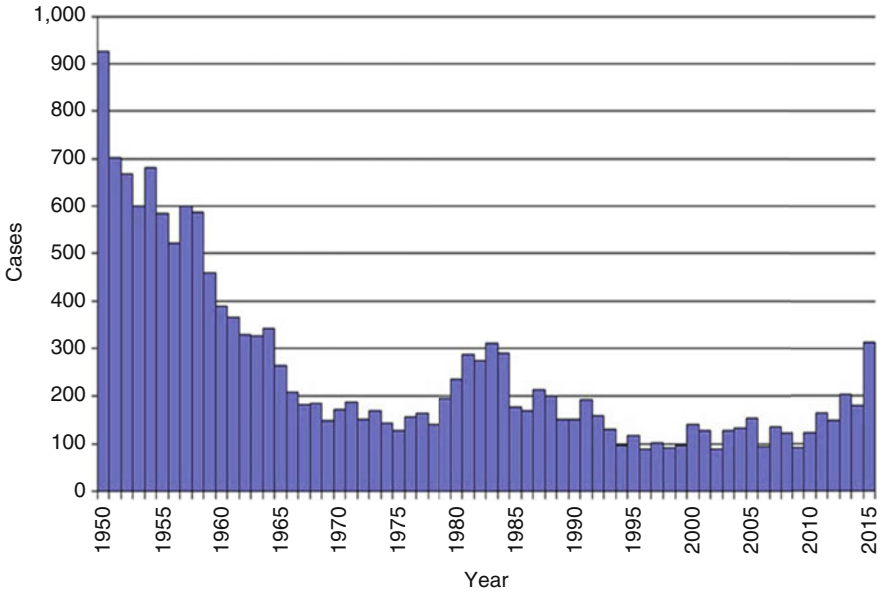

Fig. 10.2 Yearly reported cases of tularemia in the USA from 1950 to 2015. Tularemia was much more common in the earlier part of the twentieth century compared to later years. Content source: US Centers for Disease Control and Prevention

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Yersinia pestis

11

Minoarisora Rajerison and Thomas Kratz

11.1 Introduction

Plague is frequently associated with history textbooks. Nevertheless, this disease can pose a threat even today. Pneumonic plague is a deadly disease and rapidly evolving. Because of the disease characteristics, *Yersinia pestis* can play a role as a potential bioweapon. Today, plague is endemic in countries such as Madagascar and the United States. This chapter aims at sharing experiences made in plague-endemic countries as well as enhancing emergency preparedness and depicting protective measures.

11.2 Pathogen Characteristics

The genus *Yersinia*, a member of the *Enterobacteriaceae* family, includes three species that are pathogenic for rodents and humans: *Yersinia enterocolitica*, the most prevalent one in humans, causes gastrointestinal syndromes ranging from acute enteritis to mesenteric lymphadenitis. *Yersinia pseudotuberculosis* causes mesenteric adenitis and septicemia, and *Yersinia pestis* causes plague. Genetically, *Y. pestis* is a monomorphic clone of its more diverse parental species, *Y. pseudotuberculosis* [1]. These two species share a high degree of homology at the genomic level, but they differ radically in their pathogenicity and transmission. *Y. pestis* has caused three pandemics resulting in millions of deaths, whereas

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Y. pseudotuberculosis only causes a mild enteric disease that rarely leads to death [2].

Y. pestis is a gram-negative, non-motile, non-spore-forming rod-shaped bacterium (0.5–0.8 μm in diameter and 1–3 μm long) that exhibits bipolar staining with Giemsa or Wayson staining. *Y. pestis* is subdivided into the subgroups *Orientalis*, *Medievalis*, *Antiqua*, and *Pestoides*, based on their abilities to ferment glycerol and reduce nitrate [3].

11.3 Pathophysiology

Y. pestis strains carry three plasmids, each harboring important virulence mediators: pPCP1 (also called pPla), pMT1 (pFra), and pCD1 (pYV). Plasmid pCD1 encodes the low-calcium response (LCR) type III secretion system (T3SS) which is essential for *Y. pestis* pathogenesis via all routes of infection. The *Yersinia* T3SS is responsible for injecting the *Yersinia* outer proteins (Yops) into target host cells where the Yops have antiphagocytic and/or anti-inflammatory effects. *Y. pestis* initially targets alveolar macrophages in the lung through injection by the T3SS, followed by a shift in *Y. pestis* host cell preference corresponding to invading neutrophils [4]. During pulmonary infection, wild-type *Y. pestis* can promote the growth of nonvirulent bacteria via a T3SS-dependent mechanism, indicating that *Y. pestis* creates an immunosuppressive environment in the lungs by targeting professional phagocytes [5]. pMT1 (also designated as pFra) is responsible for the synthesis of fraction 1 (F1) antigen and phospholipase D. F1 antigen has been suggested to be involved in the antiphagocytic activity reported for *Y. pestis*. F1 and the virulence plasmid-encoded T3SS act in concert to make *Y. pestis* highly resistant to uptake by phagocytes [6]. Plasmid-encoded phospholipase D, previously characterized as a murine toxin, plays a major role in survival of plague bacteria in fleas [7]. The plasmid pPCP1 (pPla) encodes major virulence determinants including the protease Pla which is required for the development of pneumonic plague. Acquisition of pPCP1 is a critical step in the adaptation of *Y. pestis* to the pulmonary environment [8, 9].

11.4 Clinical Features and Incubation Period

11.4.1 Pneumonic Plague

Most frequently, lung infection with *Y. pestis* is a secondary development of a bubonic form characterized by the swelling of regional lymph nodes to a septicemic and finally pneumonic form: Bacteria reach the lung through lymphohematogenous spread [10]. Afterward, airborne transmission of the infective agent may take place via the respiratory route, resulting in primary pneumonic plague among close contacts. All persons with primary or secondary pneumonic plague can spread the disease via airborne droplets. The disease resulting from direct infection of the

airways is usually called *primary* pneumonic plague. This form occurs after inhalation of *Y. pestis* [11] that is suspended in respiratory droplets from humans or in aerosol from animals with plague pneumonia. Pneumonic plague is a severe manifestation of *Y. pestis* infection in humans, characterized by a high case fatality rate and the potential for person-to-person spread [12]. The risk of infection is higher indoors than outdoors. Environmental factors (such as low temperature, increased humidity) and crowding contribute to the spread [13–15]. Other ways of transmission include direct handling of infected animal tissues, e.g., from a *Yersinia pestis*-infected rodent (live or dead) or other animals while skinning or cutting meat, inhalation of respiratory secretions from animals (most commonly domestic cats), as well as ingestion of infective materials (infected meat that has not been sufficiently cooked to kill *Y. pestis*).

Concerning pneumonic plague, the exposure to infected animals is the most frequent source of causative infection. For example, the 1910–1911 and 1920–1921 Manchurian plague started among seasonal marmot hunters when people were in contact with sick or dead animals. Besides, hunters stayed in overcrowded underground inns in close contact [16]. Due to person-to-person transmission, 60,000 people died during the first and 9300 people during the second Manchurian plague epidemic.

From 1970 to 1993, about 2% of all plague cases in the United States were diagnosed as primary pneumonic plague cases which were contracted from infected cats [17, 18]. In 1992, a man from Tucson, Arizona, died of an illness which was diagnosed as primary pneumonic plague. His infection was acquired by respiratory exposure to a presumably *Yersinia pestis*-infected domestic cat which was reported to have submandibular abscesses and oral lesions compatible with feline plague [19–22].

A wildlife biologist working in the Grand Canyon National Park in Arizona most probably acquired pneumonic plague through inhalation of aerosols generated during a postmortem examination of an infected mountain lion: He was found dead in his residence 1 week after conducting this examination [23].

In Ankazobe (Madagascar), a rat reportedly dropped from the ceiling onto a table in front of a man. The rat was still breathing, stretched its legs, and died after a few movements. The man took the dead rat and buried it without taking any protective measures. Two days later, he suffered from fever and chest pain. Upon medical consultation, the patient received an antimalarial treatment as well as penicillin. He became thirsty and cyanotic. A second physician administered a specific treatment against plague (streptomycin), and the patient finally recovered (Andrianalimanana S, unpublished data). His sputum tested positive for F1 using rapid diagnostics but was negative on culture. According to definition of the WHO, this case was classified as presumptive case as it occurred in a plague-endemic area [24].

In Madagascar, an exceptional transmission route was reported for inhalation of *Y. pestis*: As a traditional remedy, a traditional healer incised a septicemic/secondary pneumonic plague patient's epigastric region and sucked out some blood. The patient died early the next morning. Three days later, the healer presented symptoms

of pneumonic plague and died after another 3 days. This healer then became the source of an extensive person-to-person transmission chain which was furthermore augmented through his family members (wife and son), six brothers, and other villagers. Within 16 days, 18 cases were recorded, 8 of whom died [25].

Secondary pneumonic plague only develops in a minority of patients with bubonic plague or septicemic plague—this was applicable for approximately 12% of plague cases in the United States during 60 years [26] and less than 7% in Madagascar during 10 years (anecdotal data). Statistically, secondary pneumonic plague is rarely mentioned because it is a secondary development of a bubonic or septicemic plague, with the "initial" form being notified in the declaration form. An example of a notified secondary pneumonic plague case was a woodcutter who was suspected to suffer from bubonic plague with axillary adenitis and fever. He did not receive any treatment for 6 days. After this time, he presented the clinical signs of pneumonic plague and died without having been promptly treated [25].

For pneumonic plague, the incubation period is 1–3 days, although rapid onset and death can occur in less than 24 h [27].

11.5 Routes of Infection

Plague is primarily a disease of rodents. Humans are extremely susceptible to *Y. pestis* transmission and may be infected either directly or indirectly. Transmission of plague from animal to human usually happens via the bite of an infected flea. When a flea feeds on a rodent host, blood is taken into its stomach. If *Y. pestis* are present in this blood meal, the bacteria multiply and form an obstruction at the flea's proventriculus. When the flea stops sucking, the blood-filled esophagus recoils, and the accumulated blood is driven into the new host, carrying *Y. pestis*. The time interval until a flea is infectious is dependent on the flea species and external temperature and humidity.

Y. pestis can cause bubonic, septicemic, and pneumonic plague in humans (Fig. 11.1).

The risk to humans of acquiring plague from dead infected animals is assumed to be small and can be reduced further by educating the public about avoiding sick or dead animals.

11.6 Clinical Picture

The description below of the clinical picture includes the usual clinical manifestation of plague. However, taking the individual patients' anamnesis is crucial, as additional elements such as:

- Traveling in a known plague focus
- Contact with a case of human plague
- Presence of bubo

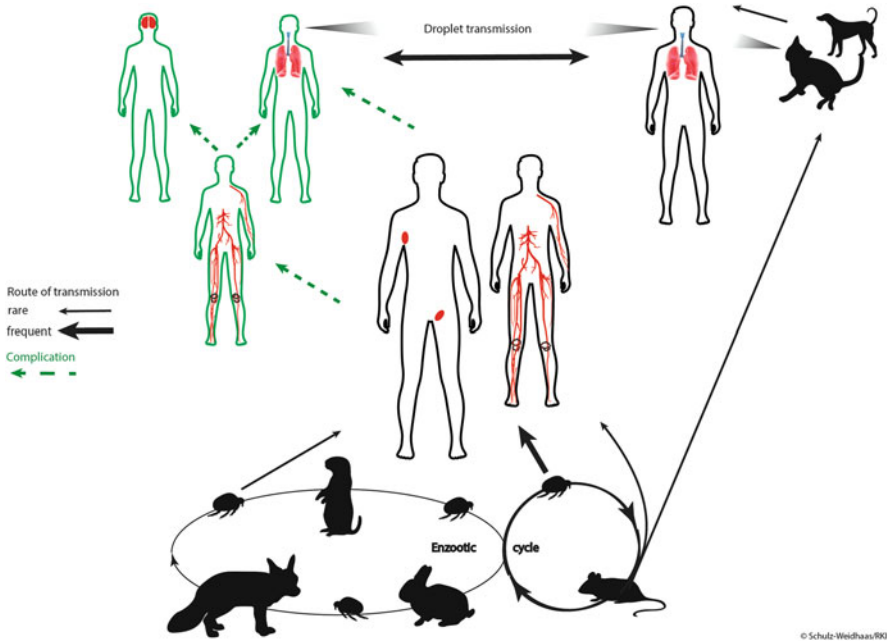

Fig. 11.1 Routes of infection and complications of plague. Primarily *Y. pestis* is transmitted from animals to humans by fleas, causing bubonic or septicemic plague. These primary forms of plague can exacerbate to secondary pulmonary plague or plague meningitis. Pulmonary plague is easily transmissible from human to human through droplet transmission and then causing primary pulmonary plague. Primary pulmonary plague can also be acquired through direct contact with coughing cats infected with *Y. pestis* or, on rare occasions, with dogs [28, 29]

– Presence of cough with blood-stained sputum
– High fever and quick onset of symptoms
– Recent use of drugs in general and antibiotics in particular

can contribute to or rule out the suspicion of plague. Patients typically experience a sudden onset of illness characterized by malaise, fever, shaking chills, and headache. Gastrointestinal complaints (abdominal pain, nausea, vomiting, and diarrhea) are possible.

11.6.1 Bubonic Plague

Y. pestis can cause bubonic, septicemic, and pneumonic plague in humans. In humans, plague most commonly presents as the bubonic form of the disease which is mostly associated with the bite of an infectious flea or, rarely, with direct contact with infectious bodily fluids or tissue of a host. Symptoms appear after an incubation period of 2–6 days which may occasionally be longer [30], with acute and

very rapid onset of nonspecific symptoms, including high fever (38–40 °C), malaise, headache, muscle aches, and sometimes nausea and vomiting. At the same time, or within 24 h, the patient notices buboes characterized by severe pain, swelling, and marked tenderness. A patient suffering from bubonic plague is distinguishable from patients with lymphadenitis from other causes by the absence of cellulitis (caused by injury or toothache), rapid onset of symptoms, and rapid deterioration of the patient's condition.

The lymph node responsible for draining the area proximal to the site of infection is affected, and a bubo develops. There is surrounding edema, and the overlying skin is warm and reddened. One or two days after, symptom onset bean-sized or slightly larger buboes can be easily identified by palpation. Palpation at this stage is usually painful or very uncomfortable for the patient who also will avoid movement, pressure, and stretching around the bubo; even contact with clothes is very painful. From the fifth to sixth day of disease onward, without effective antimicrobial treatment, bubonic plague may progress to an increasingly toxic state of fever, tachycardia, and lethargy leading to prostration, agitation, confusion, and, occasionally, convulsions and delirium. Failure of the body to filter out and kill the bacteria in the lymph node allows hematogenous spread and invasion of peripheral organs. Progression to this systemic stage of disease, termed septicemic plague, is marked by a case fatality rate of 90%. Complicated septicemic plague can be accompanied by shock, multiple organ failure, and meningitis. The terminal stage of bubonic plague is secondary pneumonic plague: *Y. pestis* colonizes the lung via the bloodstream.

When an appropriate course of antibiotics is given during the early stages of the disease, the patient usually responds quickly, and fever disappears over 2–5 days, followed by the other systemic manifestations. Nevertheless, buboes often remain enlarged and tender for a week or longer after treatment. Differential diagnoses include, among others, streptococcal or staphylococcal lymphadenitis, infectious mononucleosis (causative agent: Epstein-Barr virus), cat-scratch fever (*Bartonella henselae)*, lymphatic filariasis (helminths, e.g., *Wuchereria bancrofti*), and tick-borne typhus (*Rickettsiae* spp.). Involvement of intra-abdominal lymph nodes may mimic appendicitis, acute cholecystitis, enterocolitis, or other intra-abdominal surgical emergencies [30].

11.6.2 Pneumonic Plague

Pneumonic plague is a unique respiratory syndrome that results in a purulent, multifocal, severe exudative bronchopneumonia. It is the deadliest form of plague [27, 31]. The disease is characterized as biphasic: The early phase is relatively asymptomatic and noninflammatory, while the latter phase is highly pro-inflammatory, resulting in massive lobar lesions (see Fig. 11.2). Besides zoonotic infection, which is mainly maintained by wild rodents and fleas as the natural cycle of transmission, intentional release of aerosolized *Y. pestis* resulting in person-to-person transmission is the most feasible bioterrorism threat [13].

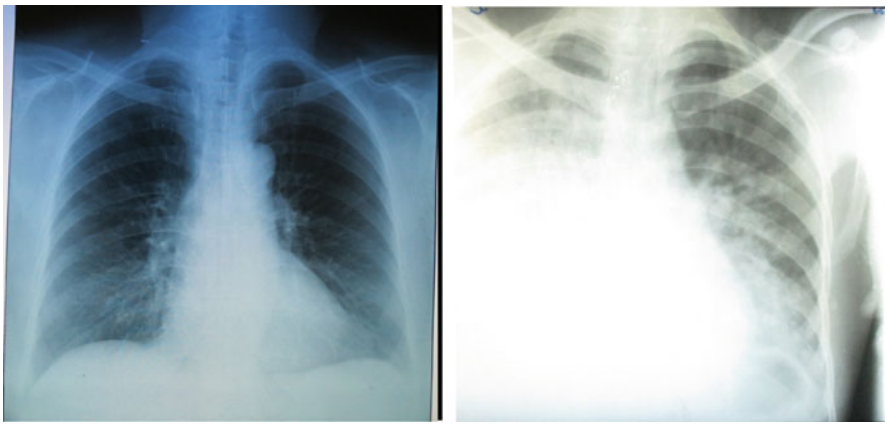

Fig. 11.2 Left: Chest X-ray of a healthy individual. Right: Chest X-ray of a pneumonic plague patient. © Plague Unit—*Institut Pasteur de Madagascar*

A primary pneumonic plague patient usually has an infectious pneumonitis at the onset of symptoms, often within 24–48 h after exposure. Physical vigor is largely intact when infection generates an intense cough reflex productive of thin serosanguineous sputum. This is readily aerosolized into droplets (<5 mm in diameter), which close contacts may inhale deep into their respiratory tract. *Y. pestis*, as invasive pathogenic bacteria, have the capacity to proliferate in the tissue of infected hosts [32]. Upon proliferation, *Yersinia* is able to inhibit the respiratory burst. The bacteria enter the lungs through the windpipe and start attacking the lungs and throat. Infection of the respiratory system by *Y. pestis* initially proceeds with a low degree of bacterial replication, and the bacteria are generally undetectable in the sputum for at least 24 h [33, 34]. Although bacterial proliferation is rapid, the initial stage is relatively quiescent [35]. In the case of primary pneumonic plague in humans, bacteria are found in the alveoli. Microscopic examination of the lung tissue reveals multiple histological patterns, including acute pneumonia, intra-alveolar hemorrhage and edema, and the presence of extracellular bacteria in the alveoli [36]. For individuals who died of primary pneumonic plague, *Y. pestis* was found mostly in the small airways and alveoli as extracellular bacteria [15, 16, 27, 37].

Secondary plague pneumonia results from the hematogenous spread of *Y. pestis* to the lungs, usually because of untreated or advanced infection of an initially bubonic or septicemic form. Many patients die before they develop well-advanced pneumonia. Those who do not die may be too sick for their cough reflex to have the strength to produce finely aerosolized droplets. Consequently, during the early stages of disease, the risk of transmission is high. Cough, sputum production, increasing chest pain, difficulty in breathing, hypoxia, and hemoptysis become prominent as the disease progresses rapidly. Clinical presentation does not differ from pneumonia of other origins; however, a rapid course of disease and high lethality are indicative of pneumonic plague. Compared to the bubonic form of the

disease, pneumonic plague results in higher rates of morbidity and lethality. The deadly nature of the disease is further exacerbated by the small window for administering effective treatment after the onset of symptoms [13]. Pneumonic plague is one of the most fulminating diseases known to mankind, and patients die if specific antibiotic therapy is not begun within the first 18–24 h after disease onset. Its case fatality rate approaches 100% if untreated [12] and is more than 50% with antimicrobial treatment [25]. Pneumonic plague is highly contagious and requires strict adherence to respiratory droplet precautions.

Bubonic plague is a *medical* emergency for its high mortality if untreated.

Pneumonic plague is a *medical* and *public health* emergency because of its high lethality and danger of airborne spread to the community.

11.7 Laboratory Diagnostics

Laboratory examination of specimens from clinically and/or epidemiologically suspected case(s) is important in order to establish the diagnosis of plague, to support appropriate preventive and control measures, to classify the case according to WHO standard definition, and to notify the case according to the revised International Health Regulations that came into effect in June of 2007.

11.7.1 Sample Collection

When plague is suspected, clinical specimens should be collected urgently, but specific antimicrobial treatment should be started without waiting for the laboratory result. All specimens indicated should be collected and transported to a reference laboratory after clinical diagnosis has been made. Precautions for handling highly infectious biological samples should be applied when taking specimens. The clinical samples, such as bubo aspirates for bubonic plague, sputum for pneumonic plague, or postmortem liver or lung biopsy for a deceased case, need to be collected and absorbed on a swab and transported in Cary-Blair medium for bacteriological confirmation to a reference lab. Blood samples could be used for culture and antibiogram of *Y. pestis*; they should be collected whenever possible. Bacteria may be intermittently released from affected lymph nodes into the bloodstream; therefore a series of blood samples taken 10–30 min apart may be productive in the isolation of *Y. pestis*.

11.7.2 Sample Packaging

According to the currently accepted US CDC biosafety norms, *Y. pestis* is listed under Biosafety Level 2 [38]. Therefore, WHO regulations for shipping dangerous goods and IATA transportation rules apply when the specimens are to be shipped via air transport either domestically or internationally. For sample packaging, Cat. A of UN 2814 should be applied [39]. Standardized packaging methods and materials ensure safety of personnel and specimen integrity, even if the package were damaged during transport. If international transport is necessary, authorization to import the specimens should be organized by the reference laboratory, which should also inform the sender of receipt or nonreceipt of the specimens.

Laboratory request forms must accompany the labeled specimens. Biohazard labels must be placed on package. Storage temperature requirements must be written on package. Absorbent-wrapped materials should be placed in a leak-proof bag, soaked with disinfectant (quaternary ammonium or phenolic solutions), sealed, and placed in proper container. The specimen container should be watertight and leak-proof. Letters, forms, permits, airway bills, and other identifying/shipping documents for the receiving laboratory should be placed together in a plastic pouch and taped onto the outer transport packaging. The transport service must also receive a copy of these documents.

11.7.3 Laboratory Examination

Appropriate specimens should be examined for evidence of plague. The gold standard is microbiological culture. A thin smear or rapid diagnostic test can be made from the sample. The specimen is inoculated onto general laboratory media and into laboratory mice for isolation and for amplifying the recovery of a pure *Y. pestis* culture. *Y. pestis* are susceptible to lysis by a specific bacteriophage. Identification of biochemical profiles should be used as a supplemental diagnostic test (see Fig. 11.3).

Smears are colored with Giemsa or Wayson stain and checked for the presence of bipolar staining gram-negative bacteria.

A rapid diagnostic test (RDT) for detecting the F1 antigen based on the use of monoclonal antibodies to the F1 antigen of *Y. pestis* has been developed, produced, and evaluated under field conditions in Madagascar. This RDT is suitable for a wide range of clinical specimens (bubo aspirate, sputum, serum, and urine) and has shown an excellent sensitivity and a great specificity compared to bacteriology and ELISA methods. This simple, rapid, and easy-to-use method is of key importance for health workers located in remote sites and for rapid diagnosis in the case of a bioterrorist attack. Indeed, its development and commercialization have contributed to better case management and surveillance in Africa. The availability of such a test in other countries with endemic plague is expected to have a similar impact [40].

For culture by bacteriology, *Y. pestis* grows on most routine laboratory culture media and needs 2 days of incubation at an optimum temperature of between 25 °C

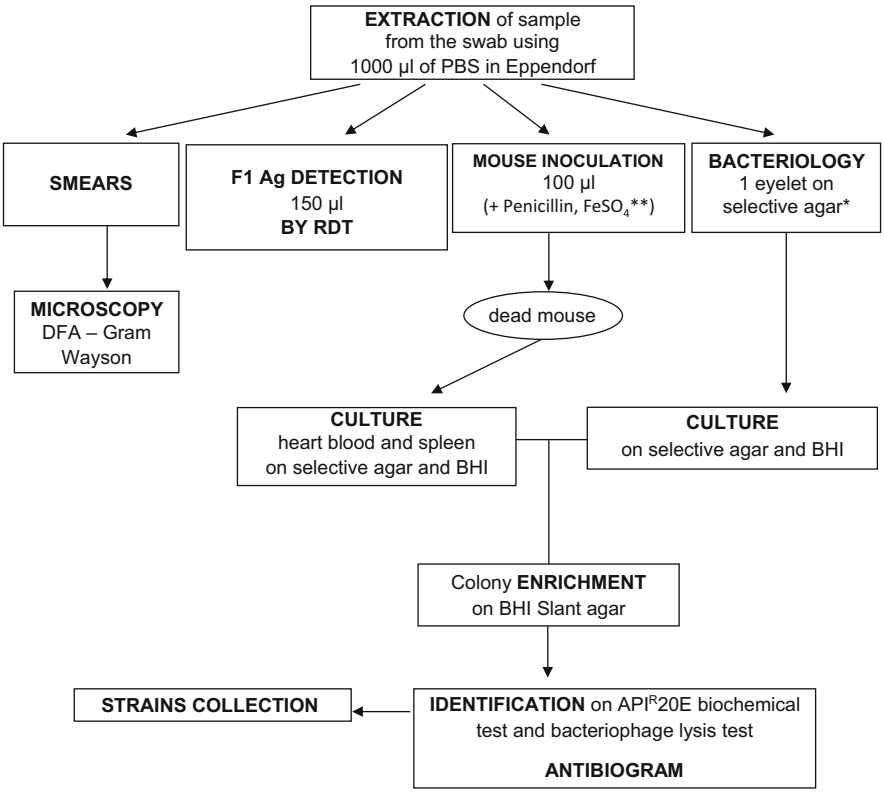

Fig. 11.3 Processing of clinical specimens in the reference laboratory. Clinical specimens conveyed in Cary-Blair agar: slip out the swab, avoid taking agar. *BHI* brain heart infusion, *DFA* direct immunofluorescence antibody, *RDT* rapid diagnostic test. Single asterisk, selective agar (CIN media) or blood agar can be used. Double asterisk, prior injection with $FeSO_4$ before inoculation with sample to be amplified in mouse

and 29 °C for achieving visible colonies. The colonies are opaque and smooth with irregular edges. In broth culture, for example, brain heart infusion or peptone broth, *Y. pestis* culture is characterized by a clear broth with clumps of cells at the bottom of the tube. An identification of *Y. pestis* can be made on the basis of biochemical tests and lysis by a specific bacteriophage.

Serology by passive hemagglutination or enzyme-linked immunosorbent assay (ELISA) may be used as confirmation tests if the pathogenic agent could not be isolated. These immunological approaches require two serum samples from the patient (acute and convalescent sera). Antibodies against the F1 antigen usually appear 1 week after the onset of symptoms. A fourfold rise in titer of paired samples is confirmatory for *Y. pestis*. Recently, RDT for the detection of plague anti-F1 antibodies in a range of reservoirs have been developed and evaluated. This is of great interest for the surveillance of reservoirs and active foci and for plague diagnosis [30].

11.8 Case Definition of Plague

According to the WHO standard definition, plague patients are classified in three groups depending on the results obtained from laboratory confirmation [24]:

1. Confirmed case if a strain of *Y. pestis* has been isolated by culture or mouse inoculation, or if F1 antigen RDT and pla PCR are both positive, or if there is a fourfold rise in anti-F1 antibody titer in paired serum samples
2. Presumptive case if one of the following tests is positive: F1 RDT, or microscopy (gram-negative bacteria showing bipolar staining which is the morphologic pattern of *Y. pestis*), or a single anti-F1 serology without isolation of *Y. pestis*
3. Suspected case if no sample is available from the patient or if all tests performed are negative

11.9 Clinical Management of Plague

11.9.1 Introduction

The occurrence of cases of pneumonic plague is a likely scenario in attacks with biological weapons. Furthermore, bubonic plague can, if untreated, lead to pneumonic plague. Untreated pneumonic plague can be fatal as early as within 24 h [41]. Antibiotic therapy should therefore be started promptly, even if a suspected case has not been confirmed yet by laboratory diagnostics. Drug-resistant forms of *Y. pestis* have been detected, though rarely, during several natural outbreaks of *Y. pestis* and should always be kept in mind [42, 43].

11.9.2 Supportive Therapy

Supportive therapy for patients suffering from severe forms such as pneumonic plague, plague meningitis, and plague sepsis implies intensive care including cardiovascular monitoring and possible ventilation in addition to specific antibiotic therapy. Gram-negative sepsis would be expected as well as its complications (adult respiratory distress syndrome, disseminated intravascular coagulation, endotoxic shock, and multiple organ failure) [13, 44]. For sepsis management, see www.survivingsepsis.org. Lack of capacities for mechanical ventilation can become a challenge: During a drill performed in the United States, the influx of just more than 400 simulating “cases” of pneumonic plague led to a shortfall in respirators in a regional hospital with 480 intensive care beds [45]. Contingency plans within the healthcare system in order to enhance treatment capacities differ largely between countries [46].

11.9.3 Specific Antibiotic Therapy

There is official guidance on antibiotic therapy through international and national recommendations, such as from the:

- World Health Organization (WHO): http://www.who.int/csr/resources/publications/plague/WHO_CDS_CSR_EDC_99_2_EN/en/
- European Medicines Agency (EMA): http://www.ema.europa.eu/docs/en_GB/document_library/Other/2010/08/WC500095413.pdf
- US Centers of Disease Control and Prevention (CDC): https://www.cdc.gov/plague/healthcare/clinicians.html

The abovementioned guidelines focus on treatment with aminoglycosides (e.g., streptomycin, gentamicin), fluoroquinolones (e.g., ciprofloxacin, levofloxacin, moxifloxacin), sulfonamides (e.g., sulfamethoxazole/trimethoprim), tetracyclines (e.g., tetracycline, doxycycline), and chloramphenicol.

On the other hand, drugs belonging to the beta-lactam group such as penicillins and cephalosporins have *not* proven to be sufficiently efficient for the treatment of plague.

In addition, when choosing antimicrobial agents for plague treatment, the following should be taken into account:

- The existence of **national guidelines** for the treatment of plague.
- The **clinical picture** of *Y. pestis* infection: A high degree of drug tissue penetration can be required when managing cases of plague meningitis, pleuritis, or myocarditis. Antibiotics meeting those needs, such as levofloxacin or chloramphenicol, should be used in those cases [44].
- Whether **singular cases or a mass casualty setting** occurs: In the latter case, oral and widely available antibiotics such as doxycycline or ciprofloxacin might be preferred [13].
- The possibility of infection with a **resistant strain** of *Y. pestis:* Resistances including those against streptomycin, chloramphenicol, sulfonamides, and tetracycline have sporadically been described during natural outbreaks [43]. An antibiogram should therefore be performed immediately as well as in the course of treatment when suspecting therapeutic failure.
- The **availability of antibiotics** in the country concerned in terms of resources and drug approval: Depending on national guidelines and drug approvals, drugs for treating plague might only be recommended or approved, respectively, for the treatment of common diseases. Other drugs, such as chloramphenicol, are not available at all in numerous countries.
- The **standard of** the **healthcare setting** (availability of hospital/intensive care capacities): Administration of antibiotics might require an intravenous catheterization entailing the need for hospitalization. Furthermore, some antibiotics (e.g., gentamicin due to its potential nephrotoxicity) require close monitoring of routine blood parameters such as renal or liver enzymes.

– **Groups at risk** (e.g., pregnant/breastfeeding women and children): In pregnant women, numerous antibiotics are contraindicated. For children, available data on antibiotic treatment is scarce. However, CDC points out that life-threatening conditions can justify the administration of otherwise contraindicated antibiotics [47].

CDC guidelines recommend treatment duration of 10–14 days or until 2 days after fever subsides [47]. Patients who received an adequate and proven effective antibiotic therapy for at least 72 h and are clinically non-symptomatic can be discharged.

11.9.4 Postexposure Prophylaxis

Antibiotic postexposure prophylaxis (PEP) is documented, such as therapy, in:

– WHO guidelines: http://www.who.int/csr/resources/publications/plague/whocdscsredc992b.pdf
– EMA guidelines: http://www.ema.europa.eu/docs/en_GB/document_library/Other/2010/08/WC500095413.pdf
– CDC guidelines: https://www.cdc.gov/plague/healthcare/clinicians.html

Doxycycline and ciprofloxacin as well as tetracycline and sulfamethoxazole/trimethoprim are mentioned in these guidelines. In close contacts of plague patients, PEP should be initiated as early as possible within the incubation period (max. 7 days) after initial exposure, with daily administration of antibiotics for 7 days.

For pregnant women and children, the administration of doxycycline or ciprofloxacin could be considered. This should only be done if the benefit outweighs the risk [47].

11.9.5 Dead Body Management

Catapulting dead bodies infected with *Y. pestis* as a means of medieval biowarfare in the fourteenth century has been described [48]. The microorganism can survive up to 2 months at 35 °C in carcasses [49]. Furthermore, dead bodies are likely to be infested with fleas and lice which can spread the disease. Healthcare worker protection including wearing personal protective equipment (PPE) should be maintained when handling a dead body. Avoidable manipulations such as traditional washing and embalming should not take place. The dead body should be placed in a body bag. Incineration of the dead body can be considered while respecting cultural sensitivities. For general advice on dead body management, please refer to the chapter on clinical management of HCID patients.

11.10 Healthcare Worker Protection

Universal precaution should be applied and personal protective equipment (PPE) worn when caring for patients suffering from plague.

Pneumonic plague is a highly contagious disease transmissible by droplets. A transmission by droplet nuclei has not been documented. Transmission typically occurs through direct and close contact. CDC determines a direct and close contact to be anyone who has been within 6 feet (~1.8 m) of a patient with plague while they were coughing up blood [50]. Infections coming from asymptomatic carriers of *Y. pestis* have not been documented so far. For further details on transmissibility of plague, see Sect. 11.5.

11.10.1 Universal Precautions

Please refer to the chapters on *clinical management of HCID patients* as well as *infection prevention and control*. Universal precautions including droplet precautions should be applied [13, 51]. In cases of pneumonic plague, the patient should wear a surgical mask in order to avoid the spread of droplets through coughing.

11.10.2 Choosing Personal Protective Equipment

Personal protective equipment (PPE) should be safe, user-friendly, and appropriate. The challenge comes from diverging national and international recommendations. For general advice, see also chapter on *clinical management of HCID patients → Personal protective equipment*. Standard operating procedures (SOP) of healthcare facilities and public health institutions should specify which PPE should be used in the case of pneumonic plague occurrence. Because of its size of 0.5–3 μm, *Y. pestis* is filtered by FFP3 respirators which are, according to DIN EN 149, challenged with a median particle size of 0.6 μm. In order to avoid leakage of face masks, either an "all in one solution" (e.g., with PAPR) should be applied or the FFP3 respirator should be tested with a qualitative or quantitative FIT test [52].

There are three types of PPE which might be applied when dealing with a patient suffering from pneumonic plague:

– PPE using a combination of face mask, goggles, and overall:
  Its use is limited in that particular agents for decontamination (e.g., peracetic acid) cannot be used due to lack of filtering of chemical agents by FFP3 respirators.

- Powered air purifying respirator (PAPR) which is frequently used, for instance, in high-level isolation wards in Europe [53].
- Self-contained breathing apparatus (SCBA) which might be applicable if, at a site of a potential bioterrorist attack, the agent is still unknown and the risk of, e.g., radiological/chemical/toxin/anthrax spore exposure cannot be excluded [54].

The choice of PPE ("classical PPE," PAPR, or SCBA) should be made based on factors such as:

- Is the agent still unknown at a site of a potential bioterrorist attack?
- Is there a hint that the involved strain of *Y. pestis* might be multidrug-resistant, hindering PEP with, e.g., doxycycline or ciprofloxacin?
- Is there a need of thorough decontamination of PPE or (e.g., in the case of arbitrary spread of aerosols) of patients, dead bodies, or objects?
- Consideration of required length of shifts versus heat accumulation in selected PPE.
- Consideration of national work security regulations, e.g., restricting length of shifts, requiring initial instructions, as well as medical screening examinations.

11.10.3 Protective Measures in Laboratories

Y. pestis can pose a significant risk in laboratories. Prior to 1950, at least ten laboratory-acquired cases were reported in the United States, four of which were fatal [38]. In 2009, a fatal accidental laboratory exposure to an attenuated strain of *Y. pestis* was documented [55]. National/international laboratory networks which connect laboratories capable of performing diagnostics of potential bioterrorist agents should be known in order to identify suitable laboratories. The main risk stems from direct contact with infective materials (e.g., body fluids), inhalation of aerosols, and fleabites. Depending on the procedures performed with *Y. pestis*, US CDC suggests using BSL-2 or BSL-3 recommendations. In Germany, *Y. pestis* is generally classified as a S-3 pathogen [56]. For further elaborate instructions on safety precautions when handling *Y. pestis* in a laboratory, see https://www.cdc.gov/biosafety/publications/bmbl5/bmbl.pdf.

11.10.4 Disinfection and Decontamination

Study data indicate that commercial disinfectants are suitable to disinfect clean surfaces contaminated with *Y. pestis* [57].

According to CFSPH [58], "*Y. pestis* is susceptible to a number of disinfectants including 1% sodium hypochlorite, 70% ethanol, 2% glutaraldehyde, formaldehyde and iodine-based and phenolic disinfectants." In Germany, RKI [59] and VAH [60]

have issued lists of bactericidal agents, including their required soaking time. Differences should be made according to the targeted area of disinfection (skin or mucous membranes, surfaces, PPE, or medicinal products).

In the case of arbitrary spread of an aerosol, there are indications that *Y. pestis* remains infective in the aerosol for only up to 1 h [13, 37]. Work protection regulations in the United States therefore do not systematically recommend environmental decontamination [61]. On the other hand, in a trial *Y. pestis* could be detected on metal surfaces up to 3 days after the spread of a contaminated aerosol. Dry surroundings seem to favor the survival of *Y. pestis* [62, 63]. The decision for or against environmental decontamination should be made carefully, taking into account, e.g., the presence of dead rodents.

11.10.5 Waste Management

According to the US National Response Team (NRT), waste should be autoclaved, incinerated, chemically inactivated, or fumigated and then tested to be sure the agents were inactivated [64]. If transport of waste contaminated with *Y. pestis* is necessary (e.g., to an autoclaving facility), Cat. A of UN 2814 should be applied [39].

How to Manage Patients with Plague

- Plague is potentially a *medical emergency* and should always be considered as one.
- As contagiousness of a patient with pneumonic plague is very high, isolation of the patient is mandatory.
- Appropriate antimicrobial treatment is vital and should be initiated at the earliest.
- Specimens for diagnostics should be obtained before initiating treatment.
- Standard patient care precautions and droplet precaution are strongly recommended for health centers/staff.

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Bacterial Therapeutics

12

Martin Richter

Today we distinguish four main mechanisms that are suitable to act as target structures in anti-infective treatment concepts fulfilling the theory of selective toxicity [1, 2].

1. Inhibition of prokaryotic cell wall synthesis
2. Disturbance of cellular membrane stability
3. Blocking of protein synthesis, and
4. Suppression of nucleic acid synthesis

All pathogens discussed in this book feature an immense potential to create disease in humans. Especially bacterial pathogens also feature intrinsic mechanisms for the purpose of survival against anti-bacterial treatment, which is commonly referred to as antibiotic resistance. Additionally, bacteria such as *Bacillus anthracis*, the causative agent for anthrax or *Yersinia pestis*, the causative agent for plague, are able to multiply rapidly within the human host and therefore quickly create a life-threatening systemic infection. For those pathogens, onset of antibiotic therapy must be fast to be successful regardless of resistance mechanisms. Altogether these features are determinants of disease severity and outcome and frequently result in an excessive reaction of the host immune system against such infections. This in turn can lead to systemic shock, organ failure and ultimately death of the human host.

In the following subchapters, specific groups of antibacterial therapeutics will be discussed that can be used to treat bacterial infections where their causative agents are often considered to have a potential for misuse, for instance in biological attacks. Sometimes the term bioterror agents is used to subsume bacteria, viruses, and biological toxins that rapidly cause severe disease and/or where the lack of specific

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treatment can ultimately lead to life-threatening disease progression. Because of the scope of this book we will discuss here specifically antibacterial treatment and in Chap. 10 antiviral treatment against this group of agents (Table 12.1). In antibiotic therapy we discuss two principles of action (1) bacteriostatic and (2) bactericidal. The bacteriostatic principle of action causes an inhibition of bacterial proliferation (e.g., by interfering with bacterial protein synthesis), which causes stasis in viability but does not directly destroy the agent structurally. The bacterial agent will then often be eliminated from the body by means of protein digestion processes. The bactericidal principle of action in turn leads mostly to a direct destruction of the agent by interfering with structural processes unique to bacteria development such as cell wall synthesis.

Table 12.1 Exemplary[a] treatment options for selected high consequence bacteria

Agent	Disease	Treatment options (group of antibiotics)
Yersinia pestis	Plague	Streptomycin (aminoglycoside) Gentamicin (aminoglycoside) Tetracycline (tetracycline)[b] Doxycycline (tetracycline)[b] Oxytetracyline (aminoglycoside) Chloramphenicol Sulfamethoxazole/Thrimethoprim (sulfonamide/diamino-benzylpyrimidine)[b]
Bacillus anthracis	Anthrax	Ciprofloxacin (fluoroquinolone)[c] Meropenem (β-lactam)[d] Linezolid (oxazolidinone) Penicillin G (β-lactam)[e] Ampicillin (β-lactam)[e] Vancomycin (glycopeptide) Clindamycin (lincosamide) Doxycyline (tetracycline) Chloramphenicol Rifampin (ansamycin)[f]
Francisella tularensis	Tularemia	Ciprofloxacin (fluoroquinolone)[c] Streptomycin (aminoglycoside) Gentamicin (aminoglycoside) Doxycycline (tetracycline)
Brucella spp.	Brucellosis	Doxycycline (tetracycline) Gentamicin (aminoglycoside) Streptomycin (aminoglycoside) Sulfamethoxazole/Thrimethoprim (sulfonamide/diamino-benzylpyrimidine) Rifampin (ansamycin) Chloramphenicol[g] Imipenem/Cilastatin (β-lactam/(R)-cystein-derivative)[g,h] Tigecycline (glycylcycline derived from tetracycline)[g] Ciprofloxacin (fluoroquinolone)[c,g]

(continued)

Table 12.1 (continued)

Agent	Disease	Treatment options (group of antibiotics)
Coxiella burnetii	q-Fever	Doxycycline (tetracycline) Ciprofloxacin (fluoroquinolone)[c] Clarithromycin (macrolide)[i] Azithromycin (macrolide)[i] Sulfamethoxazole/Thrimethoprim (sulfonamide/diamino-benzylpyrimidine) Hydroxychloroquine (chinoline)[j] Rifampin (ansamycin)[j]
Burkholderia mallei	Glanders[k]	Sulfadiazine (sulfonamide) and other sulfonamides Tetracycline (tetracycline) Doxycycline (tetracycline) Ciprofloxacin (fluoroquinolone)[c] Streptomycin (aminoglycoside) Gentamicin (aminoglycoside) Novobiocin (aminocoumarin) Imipenem (β-lactam) Ceftazidime (cephalosporin)
Burkholderia pseudomallei	Melioidosis[k]	Ceftazidime (cephalosporin) Amoxicillin/Clavulanate (β-lactam) Meropenem (β-lactam) Sulfamethoxazole/Thrimethoprim (sulfonamide/diamino-benzylpyrimidine) Doxycycline (tetracycline) Ceftriaxone (cephalosporin) Ticarcillin/Sulbactam or Clavulanate (β-lactam) Aztreonam (monobactam)

[a]Depicted treatment options are exemplary and focus on agents that have been categorized as a biological threat if used in an attack. The list is not necessarily exhaustive. Overall substance-groups in parentheses and representatives are given. Note that specific representatives may vary internationally by derivative and brand. For dosage, dosage interval, combination, length of treatment and route of administration please refer to relevant national or international guidelines (e.g. WHO). Note that some depicted substances may have only restricted approval or no approval by the respective responsible national agency. Note that intensive care/supportive therapy in addition to medical treatment improves severe disease outcome and should always be considered. In cases of severe pulmonary involvement aggressive ventilation and extra-corporal membrane oxygenation (ECMO) therapy has also been used to improve outcome

[b]Suggested for prophylaxis (pre or post exposure)

[c]Alternatively other fluoroquinolones (e.g. Levofloxacin, Moxifloxacin, Ofloxacin, Pefloxacin)

[d]Alternatively Imipenem or Doripenem

[e]Alternatives if strain is penicillin susceptible

[f]May be considered in combination with other antimicrobials

[g]Second line antibiotic

[h]Cilastatin itself shows no intrinsic antibiotic activity but increases β-lactam plasma levels by inhibiting metabolization

[i]Alternatively; cave: some strains show resistance

[j]For long term (up to 4 years) therapy of chronic infections in combination with doxycycline or fluoroquinolone to enhance susceptibility

[k]Treatment options interchangeable

12.1 β-lactam Antibiotics and Antibiotics that Interfere with Cell Wall Synthesis

The group of β-lactam antibiotics is comprised of four subgroups (1) penicillins, (2) cephalosporins and carbacephems, (3) carbapenems and (4) monobactams. Structurally unique to all of these subgroups is their β-lactam ring, which mimics the peptide D-alanyl-D-alanine. This peptide is recognized by the bacterial enzyme transpeptidase which is responsible for connecting peptidoglycan strings that function as backbones for the murein layer in the last step of bacterial cell wall synthesis. β-lactam antibiotics structurally compete with D-alanyl-D-alanine and are equally recognized by the transpeptidase. Once recognized, the β-lactam ring opens and binds covalently to the active center of the transpeptidase [3–5]. This reaction blocks the enzyme irreversible, leads to permanent incapacitation of enzyme activity, and ultimately to bacterial lysis. β-lactam antibiotics are therefore also referred to as suicide substrates. Equally, β-lactam antibiotics also interfere with bacterial carboxypeptidases [5].

Penicillins are still today among the first line choice of antibiotics if bacterial agents are susceptible to penicillin treatment. This can be attributed to practically non-toxicity of penicillins to the human host aside from penicillin allergies that can be very severe up to lethal anaphylactic shock. Physicians therefore need to ensure that penicillin allergies have not been reported in the patient's treatment history and pay close attention to strict indication and application (preferably orally or parenterally). If bioterror-relevant bacterial agents are not susceptible to penicillins other β-lactam antibiotics such as carbapenems, can be included into the treatment lineup. Side effects are comparable.

Another group of antibiotics relevant for treatment against bioterror agents that also interfere with cell wall synthesis are glycopeptides. Especially vancomycin is a representative of this group, which can be an option in treatment regimens against *Bacillus anthracis* infections (Table 12.1). Other representatives of this group of antibiotics as part of treatment options and regiments are also given in Table 12.1.

12.2 Inhibitors of Prokaryotic Protein Synthesis

This group of inhibitors is comprised of larger molecules with the exception of oxazolidinones and chloramphenicol. Relevant for treatment against bioterror agents are aminoglycosides, such as streptomycine; tetracyclines such as doxycycline; lincosamides, such as clindamycin; oxazolidinones, such as linezolid; and chloramphenicol. All of these molecules interact with 30S subunits of bacterial ribosomes, although at different stages of protein synthesis. Modes of action are (1) blocking of the acceptor position that prevents binding of aminoacyl-tRNA (aminoglycosides, tetracyclines), (2) reading error between codon and anticodon (aminoglycosides), (3) inhibition of peptide chain elongation–transpeptidization (chloramphenicol), (4) inhibition of translocation; that is the shift of peptide or amino acid carrying tRNA to its donor side (macrolides such asazithromycin, clarithromycin). The result

is always complex inhibition of bacterial protein synthesis. Note that anaerobic bacteria do not accumulate aminoglycosides and are therefore per se resistant against this subgroup of antibiotics. In addition, resistance genes that code for aminoglycoside deactivating enzymes exist in some aerobic bacteria. Most of those bacteria are able to share this resistance by transferring resistance plasmids to susceptible bacteria of the same or even different bacterial species horizontally.

Besides moderate side effects, such as gastrointestinal disorders, some more severe side effects that require discontinuation of treatment have also been reported for this group of antibiotics. Those can be acute colitis, nephrotoxicity, ototoxicity, significant photosensitivity with edema and shock, and allergic reactions that can result in lethal damage of bone marrow.

12.2.1 Antimycobacterial Agents: Ansamycins (Rifamycins)

The most common representative of this group of antibiotics is rifampicin also known as rifampin. It was initially used in treatment regiments against mycobacterial diseases such as tuberculosis and leprosy but is also effective against *Legionella* spp. and *Bacillus anthracis* and can be considered in combination with other antibiotics in the treatment of Legionnaire's disease and anthrax, respectively. In addition, rifampicin effectively inhibits vaccinia virus [6]. Rifampicin has a macromolecular cyclic structure and is derived from rifamycin B, a macrocyclic lactam that was isolated from *Amycolatopsis* cultures (especially *Amycolatopsis mediterranei*) [7].

Ultimately, ansamycins can be considered as inhibitors of prokaryotic protein synthesis and could be grouped there. However their antimycobacterial spectrum and unique mechanism of action has led to an outside grouping within the antimycobacterial agents. The mechanism of action of ansamycins is inhibition of prokaryotic DNA-dependent RNA polymerases by high affinity binding to the enzyme's β-subunit, thus preventing RNA transcription by blocking elongation and subsequently protein synthesis [8–10].

Ansamycins are usually well tolerated. Side effects can include gastrointestinal disorders and disorders in renal function. Allergic reactions to this group of drugs have also been recorded and ansamycins have shown a teratogenic potential in animal experiments [11] and are therefore generally contraindicated during pregnancy.

Rifampicin also exerts immunosuppressive effects. Molecular targets for this action have not yet been identified [12, 13].

12.3 Fluoroquinolones

This group of antibiotics is also referred to as gyrase-inhibitors and was derived from a nalidixic acid backbone that itself already has limited antibiotic activity. Fluoroquinolones feature a true quinolone framework and are fluorinated at the C6 or C7 position of the all-carbon ring. Practically all of them represent second

generation gyrase inhibitors with significantly improved antibiotic spectrum, less potential for resistance development and better biopharmaceutical performance compared to first generation gyrase inhibitors. Only fluoroquinolones are gyrase inhibitors that are relevant for treatment of bacteria that could potentially be used in a biological/bioterror attack. Fluoroquinolones have been classified into four groups; (1) oral fluoroquinolones with indications essentially limited to urinary tract infections; (2) fluoroquinolones with broad indications for systemic use; (3) fluoroquinolones of improved activity against gram-positive and "atypical" pathogens; (4) fluoroquinolones with improved activity against gram-positive and "atypical" pathogens and anaerobes [14]. Except for group (1), all other groups contain representatives with significant relevance in treatment of bacterial diseases that could be present in the population after a biological attack (Table 12.1).

Mechanism-of-action, as the name implies, lies in inhibition of DNA gyrase (topoisomerase II). Activity has also been shown against bacterial topoisomerase IV. The inhibition of these enzymes ultimately prevents proper DNA replication, which leads to a rapid breakdown of bacterial metabolism and death. In more depth: bacterial topoisomerase II catalyzes the DNA supercoiling step that is responsible for compact packaging of chromosomes in cells and bacterial topoisomerase IV catalyzes separation of newly synthesized strands of nucleic acid during replication. Inhibition of these enzymes therefore leads to improper chromosome packaging and metabolic breakdown, respectively.

Resistance development is vastly reduced in comparison to first generation fluoroquinolones but still occurs. Resistance is established by diminished susceptibility of the DNA-gyrase or a limited permeability of these anticiotics through the bacterial cell wall or both.

Fluoroquinolones are generally well tolerated. Side effects may be gastrointestinal disorders or allergic reactions to the drug. During long-term use, damage of cartilage and tendinopathy have been reported. Rarer side effects can be disorders of the central nervous system (e.g., vertigo, headache, depression).

12.4 Aminocoumarins

Another rather novel class of DNA gyrase inhibitors is the class of aminocoumarins, which have been isolated from *Streptomyces* species. The antibiotic potential of coumarins as gyrase inhibitors has been described in the early 90s [15]. Aminocoumarins prevent ATP binding to DNA gyrase, which in turn is thus not activated. Although substances from this group exert antibiotic effects, they also are highly toxic and mutations within the DNA gyrase may lead to resistance. The only relevant drug in antibiotic treatment regimens from this group is novobiocin. Novobiocin is FDA-approved and with respect to biological attacks can be used in treatment of glanders.

12.5 Inhibitors of Folic Acid Synthesis

12.5.1 Sulfonamides

The first sulfonamide (Sulfachrysoidin—Prontosil®) was introduced in 1935 by Domagk. It is therefore together with Salvarsan® and the penicillins among the first antibiotics used to treat bacterial infections in humans. Sulfonamides have increasingly lost significance over time due to their potential for causing substantial side effects and advanced resistance development. Among bacterial agents considered relevant for misuse during a biological attack, sulfonamides however are still relevant for treatment and prevention of plague and glanders.

During *in vivo* synthesis of dihydrofolic acid in bacteria, sulfonamides competitively replace para-amino benzoic acid (PABA), which is a necessary metabolite in the synthesis process. Note that humans cannot synthesize folic acid or its derivative. Humans can only cover their daily folic acid requirement by supplementing it through diet. Hence this mode of action is another practical example for the principle of selective toxicity (see page xx). Sulfonamides act as anti-metabolites and therefore suppress *in vivo* synthesis of dihydrofolic acid in bacteria and some protozoans. Since the process is competitive replacement, relatively large doses of sulfonamides need to be administered to completely replace all PABA. In addition, a lack of PABA in bacteria only leads to bacteriostasis.

Common side effects are lack of appetite and nausea. Very rarely severe side effects such as Lyell-syndrome and the related Stevens-Johnson-syndrome, both severe allergic reactions of the skin, have been described. Further, hemolytic anemia and hemorrhagic diathesis are also considered severe side effects during sulfonamide treatment.

12.5.2 Diamino-Benzylpyrimidines

Trimethoprim is the most common diamino-benzylpyrimidine representative that can be suggested in treatment line ups against agents with bio-terror or bio-warfare potential (e.g., *Burkholderia pseudomallei*, *Yersinia pestis*). Due to rapid resistance development during monotherapies with trimethoprim, it is usually administered in a combination with sulfonamides that have similar pharmacological characteristics such as sulfamethoxazole or sulfadiazine in readily available pharmaceutical formulations. Optimal synergism is achieved with a 1:20 ratio between trimethoprim and sulfonamide. Dissimilar pharmacokinetics and distribution characteristics in the human host need to be considered and lead to different initial ratios in the actual drug formulation to finally reach the desired 1:20 ratio at the target site (example combinations: trimethoprim:sulfamethoxazole = 1:5; trimethoprim:sulfadiazine = 1:2.5) [1, 2]. Substances are effective against numerous aerobic gram-positive and gram-negative bacteria. Combination with sulfonamides as described above not only delays resistance development but also increases activity range and effectivity.

Mechanism-of-action is specific inhibition of dihydrofolic acid reductase. This process ultimately prevents tetrahydrofolic acid synthesis, which is adamant for cell division processes. Note that dihydrofolic acid reductase is also present in mammals. However, diamino-benzylpyrimidines have a higher affinity to the prokaryotic analog of the enzyme by one order of magnitude and therefore induce only very minimal to negligible toxicity in humans.

Recorded side effects are nausea, exanthema, and pathological alterations to the hemogram. Because of teratogenic effects due to lack of folic acid, diamino-benzylpyrimidines and sulfonamides are contraindicated during pregnancy.

12.6 Immunotherapy Against Infections with Bacterial Agents of Bio-Warfare/Bio-Terror Concern

Especially in prophylaxis against several bacteria which are considered relevant with regards to biological attacks, some licensed and experimental vaccines exist that offer a certain amount of protection against infection, for instance adsorbed anthrax vaccine (AVA; BioThrax®, Emergent Biosolutions Inc., Rockville, Maryland, USA). Vaccination, however, offers rarely protection after exposure/infection of an individual because of short incubation periods and rapid disease progression induced by many agents discussed here. Additionally, since vaccines are not traditionally considered a classic treatment but rather prophylaxis and follow distinct immunogenic mechanisms-of-action, relevant representatives are discussed in the agent-specific chapters of this book.

In very rare cases are there options for immunological-based treatment (immunotherapy) after agent exposure or infection. These options are usually confined to passive immunization in terms of administration of antisera or specific antibodies. For example, in case of systemic anthrax infection or suspicion thereof, an antibody-based antitoxin is available for treatment in conjunction with an appropriate antibiotic regimen.

The mechanism-of-action lies in interfering with immuno-pathogenesis after infection with *Bacillus anthracis*. In general, two virulence factors have been identified in anthrax pathogenesis; (1) the bacillus capsule prevents phagocytosis and (2) AB-type exotoxins, namely lethal toxin (LT) and edema toxin (ET) [16, 17]. Further, two moieties (A and B, respectively) promote pathogenesis. The B moiety, namely protective antigen (PA), is required for cell binding, which subsequently allows the enzymatic A moieties, namely lethal factor (LF) and edema factor (EF), to enter cells in which they interfere with intrinsic immune cell recruitment by prohibiting proper immune cascade signaling and phagocytosis resulting in disruption of homeostasis and edema [18–21]. There are two available antitoxins that have been considered as treatment options, raxibacumab (GlaxoSmithKline, London, UK) and Anthrax Immune Globulin Intravenous (AIGIV) (Cangene Corporation, Winnipeg, Manitoba, Canada) [22]. Both bind to PA in a dose-dependent manner and effectively inhibit binding of PA to anthrax toxin receptors [22–24]. This action prohibits cell entry of the A moieties and ultimately LT and

ET. Raxibacumab is a monoclonal recombinant and humanized antibody, whereas AIGIV is a polyclonal serum derived from persons previously immunized with AVA [22, 24]. Both monoclonal and polyclonal treatment approaches seem to be equally effective and so far there is no evidence to suggest preferential use of one versus the other [22, 23].

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Advances in Molecular Diagnostic Approaches for Biothreat Agents

13

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13.1 Introduction

Bioterrorism is the purposeful and intentional delivery of bioweapons by non-state actors to harm humans, livestock and agriculture. The Biological Weapon Convention (BWC) was established in 1972, banning the development, production and stockpiling of microorganisms and toxins that can lead to mass destruction. The Center of Disease Control and Prevention classified bioterrorism agents into three major categories: Category A, B and C. The categorization depends upon the following criteria: (1) the easy dissemination (person-to-person) of biologically active infectious agents, (2) a major public health concern and causing a high case fatality rate both in humans and/or livestock, and (3) the creation of panic and terror among people [1].

Prior to the development of high-end diagnostic tools, basic microscopy techniques used to be a primary tool in the diagnosis of disease. Microscopy played a pivotal role in diagnosis of bacterial pathogens and helped to enumerate pathogens in different samples (e.g., blood, urine, sputum, stool). Different stains (e.g., gram-stain, Rhodamine, Indian ink) on histopathological specimens differentiated and classified several bacterial strains. The non-cultivable viruses, like rotaviruses, hepatitis A virus, and Norwalk virus, were also identified through microscopic techniques. Diagnosis was based on the morphological features of bacteria and virus particles, which required large amounts of samples, In the case of viruses, a high viral titer (10^5 to 10^6 virus particles/ml) was needed. In addition, sample losses were high during sample preparation (electron microscopy, EM), either due to sample dehydration or charging of biomolecules (bacteria and viruses), which used to result in reduced contrast and performance. Further, microscopic techniques

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required expertise and trained manpower. Advancement in the technology led to the development of transmission electron cryomicroscopy, for which specimens are preserved at −150 °C for maintaining the amorphous texture and for avoiding any ice-crystal damage. Nonetheless, the technique requires a high amount of sample (~1 mg/ml of virus particles). In addition, bacteria are far too thick for transmission electron cryomicroscopy to allow resolution of structural details [2–4]. The amalgamation of EM with cell culture practices greatly contributed in the diagnosis of bacterial and viral pathogens.

Immunochromatographic assays, polymerase chain reaction (PCR) and enzyme-linked immunosorbent assays (ELISAs) have been developed for the detection of biothreat agents. With different types of immuno-based and chip-based biosensors, biothreat agents are quickly and sensitively detected. Scientists are encouraged to use nucleic acid amplification tests (NAATs), for real-time detection of DNA and RNA using fluorescent dyes through multiplex polymerase chain reaction (PCR) assays, in which multiple pathogens can be diagnosed in a single run. PCR amplification cannot produce a large amount of high-throughput data, but next-generation sequencing (NGS) platforms provide an unbiased-in depth analysis of near-complete genomes. Molecular diagnostic techniques (Microarrays, PCR and NGS) are time saving and precise in the detection and discovery of micro-organisms in a given sample. Development of quantitative, multiplex platforms is imperative for the specific diagnosis of such pathogens, and these platforms can be easily used as point-of-care (POC) molecular diagnostic tools. With automation and miniaturization, microfluidics-based chips can be easily used as a POC platform. In addition, microfluidics integrated with various other techniques such as PCR, ELISA, and isothermal amplification enhance the sensitivity and specificity of the POC platform within financial and time constrains. These molecular diagnostic tools can be used in surveillance programs intended for biodefense preparedness.

13.2 Latest Diagnostic Techniques

13.2.1 Immunological Methods

13.2.1.1 Lateral-Flow Immunochromatographic Assays

Detection of biothreat agents by (monoclonal or polyclonal) antibodies is a standard approach in clinical diagnosis. The basic principle is the formation of an antigen-antibody complex on a solid surface followed by the visual readout. These tests are qualitative and semi-quantitative (intensity of color readout), quick (less than 20 min), cost-effective, user-friendly, and require minimal settings. Therefore, these assays are an attractive tool for POC diagnosis/prognosis in a surveillance program.

Immunochromatographic lateral flow assay are used for the detection of pathogens. Gold nanoparticles or carbon/silver/magnetic beads, upconverting phosphors and latex colored bead capture detector antibodies (a detector antibody detects the presence/absence of an analyte in a given sample) on a dry solid surface.

Once the diluted specimen is added, the detector antibodies form a complex with the cognate antigen, and the color readout indicates the test result [5–7]. A multiplexed suspension-based immuno-array can detect five biothreat agents (i.e., *Yersinia pestis*, severe acute respiratory syndrome coronavirus [SARS-CoV], staphylococcal enterotoxin B (SEB), ricin) in powder samples with a limit of detection (LOD) of 111 colony forming units (CFU)/ml, 20 CFU/ml, 110 pg, 5.4 ng, and 2 ng respectively [8].

Many rapid diagnostic kits (RDKs) are available for the detection of biothreat agents. The ENVI Assay System Gold (Environics) is a lab-in-a-box device that detects various biothreat agents within 20 min [9]. New Horizon Diagnostic Inc. offers detection kits available for various biothreat agents in patient, environment, food, and water samples [10]. Response Biomedical Inc., developed RAMP® (Rapid Analyte measurement platform) for the detection of influenza A (FluA) and B (FluB) viruses, *Bacillus anthracis*, variola virus, and ricin and botulinum toxins. This platform consists of a fluorescent reader and a disposable single-use cartridge that provides specific on-field detection of pathogens [11]. Similarly, GenPrime developed a bio-detection system for the detection of US Centers for Disease Control and Prevention-listed bioterrorism agents along with others agents [12] (Table 13.1).

13.2.1.2 Enzyme-Linked-Immunosorbent-Assay

ELISA is the most widely used method detecting enzyme-linked antigen-antibody complexes on a solid phase. The technique is user-friendly, sensitive, specific, and cost-effective [14]. The method quantifies the antigen/antibody complexes in a given sample and is used as an *in-vitro* diagnostic tool in clinical laboratories.

Different types of ELISAs are in use for the pathogen detection such as: sandwich, direct, indirect, and competitive ELISAs. These different formats detect biothreat agents such as *F. tularensis*, *B. anthracis*, *Y. pestis*, *Brucella abortus*, *Burkholderia pseudomallei*, Ebola virus (EBOV), or Marburg virus [15–21].

The integrative multiplex assay and sampling system has been reported to identify eight biothreat agents (*B. anthracis*, *F. tularensis*, *Y. pestis*, Brucella spp., *B. mallei*, ricin toxin, botulinum toxin A/B, and SEB) within 15 min. The assay is a coded strip containing immobilized antibodies to identify these agents from surface, powder or liquid samples (http://www.bbidetection.com/products/biothreat-detection-imass-device/).

13.2.1.3 Time-Resolved Fluorescence Immunoassay and Immunomagnetic Separation Electrochemiluminescence Assay

Time-resolved fluorescence immunoassay (TRF) is a version of immunoassay with extended fluorescence decay time. The long fluorescence signal helps to measure the signal after the background noise has subsided. TRF assays can detect botulinum toxin in patient samples at low concentrations (0.01 pM) [22]. The commercialized platform, dissociation-enhanced lanthanide fluorescence immunoassay, was developed by Perkin-Elmer for the detection of various pathogens. The test is similar to ELISA: a 96-well plate is coated with streptavidin and Europium- (Eu^{3+}, lanthanide

Table 13.1 Different platforms for detection of pathogens and toxins

	Product	Company	Pathogens/Toxins detected	Total run time
1.	NIDS® handheld biothreat assay and handheld reader	ANP technologies	*Bacillus anthracis*, vaccinia virus, Brucellae, Venezuelan equine encephalitis virus, *Listeria*, SEB, *Francisella tularensis*, botulinum toxin A, *Vibrio cholerae*, *Escherichia coli* 0157, ricin, *Coxiella burnetii*, *Yersinia pestis*, *Salmonella* sp.	15 min
2.	PRO STRIPS 5 Agent biowarfare threat detection kit	ADVNT Biotechnology	*B. anthracis*, ricin, botulinum toxin A and B, *Y. pestis* and SEB	Not specified
3.	Zephyr	PathSensors, Inc.	*B. anthracis*, *Y. pestis*, ricin, *F. tularensis*, orthopoxviruses, *Salmonella* sp.	15 min
4.	PrimeAlert	GenPrime, Inc.	Ricin, botulinum toxin, SEB, *F. tularensis*, *Y. pestis*	15 min
5.	ENVI Assay System Gold	Environics Oy	Ricin, botulinum toxin, SEB, orthopoxviruses, *B. anthracis*, *Y. pestis* and *F. tularensis*	<20 min
6.	Aegis 1000	BioDetection Instruments (BDI)	Foodborne pathogens, toxins, infectious agents, protein biomarkers, waterborne pathogens	<30 min
7.	RAMP 200 Biowarfare Detection System	Response Biomedical Corporation	*B. anthracis*, ricin, botulinum toxin, variola virus	>30 min

Adapted from http://www.cbrnetechindex.com/Biological-Detection/Technology-BD/Immunological-BD-T/Lateral-Flow-Hand-Held-Immunoassay-BD-I [13]

series) tagged detector antibody. The immobilized Eu-tagged antibody produces a fluorescent signal when it interacts with the antigen and releases Europium. The limit of detection of the system is 4–20 pg/ml [23].

In the IMS-ECL assay, the immunomagnetic separations are coupled with electrochemiluminescence assay (ECL) for the rapid detection of biothreat agents, *B. anthracis*, SEB toxins, and *Clostridium botulinum* in clinical specimens within 1 h with the LOD ranging from 1 pg/ml to 100 pg/ml [24].

13.2.2 Biosensors

A biosensor is an analytical device integrated with biologically active components, bioreceptors, and transducers for detection of analytes in a given sample. Bioreceptors may be enzymes, antibodies, single-stranded DNAs (ssDNA), aptamer proteins, or cells. Methods for detection of pathogen/toxins using biosensors are rapid, sensitive, and cost-effective. Immunoassays-based biosensors detect specific antigens in patient sample(s) or identify biomarkers for studying the host-immune

response during infection [25]. Scientists using biosensor(s) may opt for either label-free assays or labeled assays for detection of an analyte. A label-free assay detects the presence of an analyte directly through a transducer that can be an optical, electrical or mechanical [26]. In contrast, labeled assays use a second detector coupled with an enzyme, fluorophore or radioisotope to detect an analyte [14, 27–29].

The integrated multiplexed microarray biosensor CombiMatrix ElectraSense microarray detects *Y. pestis*, *B. anthracis*, and SEB. The LOD for SEB and *Y. pestis* is 5 pg/ml and 10^6 CFU/ml, respectively [30]. The electrochemical multiplexed immunosensor with the indirect labeled assay can diagnose *F. tularensis* infection with a LOD of 1000 CFU/ml within 25 min [31]. Furthermore, a piezoelectric immunosensor identifies *F. tularensis* with an LOD of 10^5 CFU/ml within 5 min [32].

Biosensor-based detection of EBOV glycoprotein ($GP_{1,2}$) is based on surface plasmon resonance and a quartz crystal microbalance sensor [33]. The on-paper gene circuit and visualization platform detects small molecules and RNA and strain-specific EBOV molecular patterns. This platform is cost-effective, quick and sensitive and can be used in industry, research, and biodefense programs [34].

A cell-based biosensor named CANARY (Cellular Analysis and Notification of Antigen Risks and Yields) developed at Massachusetts Institute of Technology detects emerging disease pathogens or biothreat agents relevant in sectors such as biodefense, agriculture, and food safety. Within the CANARY biosensor, engineered B lymphocyte cells express a calcium-dependent bioluminescent protein, aequorin, coupled with an antigen-specific membrane-bound antibody. The antigen-antibody binding activates an intracellular calcium ion channel, and aequorin will emit light [35, 36]. The, CANARY technology was commercialized by PathSensors Company for developing the BioFlash-E Biological Identifier and by Zephyr for screening liquid and powder samples for biological threat agents and toxins. The biosensor is a stand-alone, state-of-the-art device that can be used for both indoor and outdoor applications with an LOD of less than 100 CFU/ml within 5 min and 2–15 min, respectively [35]. The various types of advanced biosensors and their applications in the detection of biowarfare agents and infectious pathogens were extensively reviewed elsewhere [37]. The various types of biosensors used for the detection of biothreat agents are listed in Table 13.2.

13.2.3 Nucleic Acid-Amplification-Based Techniques

The development of PCR by Kary B. Mullis in 1987 revolutionized molecular diagnostics in clinical laboratories. PCR rapidly detects both biothreat agents and other pathogens in small patient samples in minimal time without compromising sensitivity or specificity. The basic principle of PCR is the isolation of nucleic acid (DNA, RNA) from a given sample and amplification by using a set of primers and thermostable polymerases. Later, the amplified products can be analyzed on gel electrophoresis or by using fluorescence-based detection systems.

Table 13.2 Biosensors for detection of biothreat agents

S. No.	Transducer	Biothreat agents	Limit of detection	Readout time	Samples tested	References
1	Electrochemical biosensor with gold-nanoparticle	Botulinum neurotoxin type E	10 pg/ml to 10 ng/ml	65 min	Orange juice and milk	[38]
2	Impedometric biosensor with gold nanoparticle	*Brucella melitensis*	4×10^5 CFU/ml	1.5 h	Milk	[39]
3	Electrochemical biosensor with bimetallic gold and palladium nanoparticles on boron-nitrite nanosheets	*B. anthracis* surface array proteins	5 pg/ml to100 ng/ml	1 h	Cell culture	[40]
4	Surface plasmon resonance and electrochemical impedance spectroscopy	*Brucella abortus*	0.05 pM	10 min	Cell culture	[41]
5	Surface plasmon resonance with antibody against F1 antigen	*Y. pestis*	10^6 CFU/ml	1 h	Environmental samples	[42]
6	Surface plasmon resonance coupled with electrochemical impedance spectroscopy	Botulinum neurotoxin A	0.045 fM		Cell culture	[43]
7	Piezoelectric Immunosensor coupled with quartz crystal microbalance (QCM) and covalently immobilized *F. tularensis* antigen	*F. tularensis*	5×10^6 cells/ml	35 min	Cell culture	[44]
8	Quartz crystal microbalance	staphylococcal enterotoxin A (SEA)	0.02 mg/L	25 min	Milk	[45]

Originally developed PCR was used for virus detection in early 1990s [46]. However, post-amplification contamination and false positives were major drawbacks. New and improved versions of PCR have been introduced, called Nucleic Acid Amplification Tests (NAAT). Following the detection of SARS-CoV in 2003 and swine influenza A (H1N1) virus in 2009, NAAT has become an integrative part for clinical diagnostics and detection of bio threat agents [47, 48]. Real-time PCR, quantitative-PCR (q-PCR), and reverse transcription-PCR (RT-PCR) are widely used for the detection and quantification of pathogens [49]. The q-PCR platform quantifies the amount of amplified product in "real-time" by using fluorescent dyes such as SYBR green and light emission upon extension primers, or using probe-based detection systems such as TaqMan™, scorpion probes, and molecular beacons. In RT-PCR, the complementary-DNA (cDNA) copy is synthesized by using reverse transcriptase, which can be amplified later by using gene-specific primers and polymerase enzyme. The human and avian influenza A viruses can be detected in patient samples by using RT-PCR [50, 51].

TaqMan probes have been developed by combining gene specific primers with fluorescently labeled probes. TaqMan chemistry employs the 5′-3′ exonuclease activity of Taq polymerase, which cleaves the probe-containing reporter and quencher [52]. TaqMan-based qPCR panels detect and quantify filoviruses, arenaviruses, and hantaviruses with an LOD of 0.001–10 PFU/PCR [53, 54].

Although probe-based or non-probe-based methods are commercialized for pathogen detection the detection of RNA viruses is still a challenging task. The error-prone replication of RNA viruses results in changes in target gene sequences, making standard primer/probes ineffective over time. Consensus PCR assays based on primers and probes with wobble codes (degenerate primers) can detect various biothreat agents such as paramyxoviruses in bats and rodents [55, 56].

In nested PCRs, two sets of primers (hemi-nested and fully-nested) are used for amplification. This method detected influenza A virus in 2001 [57, 58]. Several laboratories do not use nested-PCR due to increased work load with double amplification process and higher chances of contamination [57, 59].

Multiplex RT-PCR has been increasingly used by diagnostic laboratories for the detection of multiple pathogens in a single run. The technique is efficient and less time-consuming than singleplex PCR, even in detecting co-infections in patient samples. This technique minimizes false positives, is highly sensitive, and aids in better diagnosis and prognosis than singleplex PCR. The multiplex RT-PCR platform detects and differentiates between the influenza A, B and C viruses [60].

During the Operation Iraqi Freedom 2003, US Marines troops were infected with multiple *Shigella* sp. and Norwalk virus, resulting in an outbreak of gastroenteritis. Multiplex-RT-PCR, enzyme immunoassay and sequencing technologies confirmed the presence of these pathogens [61]. The novel set of primers and probes are used with an LOD of >95% in *in-vitro* transcribed RNA technique [62]. The multiplex qRT-PCR platform detects EBOV and Marburg virus by using conserved regions of the genomes of these viruses with the sensitivity of 28.6 copies/μl and 30.5 copies/μl, respectively [63]. The outbreak of swine influenza in 2009 led to the development of multiplexed PCR-microfluidics and a silicon nanowire module for sequencing.

These POC devices have a sensitivity of 20–30 μg/μl to detect H1N1 influenza A virus in samples [25]. In addition, the World Health Organization (WHO) licensed RT-PCR for detection of Middle East respiratory syndrome coronavirus in 2012 [64]. Nguyen et al. [65] developed a multiplex PCR assay for the detection of *E. coli*, *Salmonella* and *Listeria monocytogenes* in patient samples and food samples with the sensitivity of 10 CFU/ml [65].

Many multiplex PCR kits have been developed for the quick detection of various biothreat agents. Altogether, the sensitivity and specificity of multiplex PCR assays are very good, and the assays can be used in routine diagnosis [66]. The Food and Drug Administration (FDA) licensed the use of the xTAG® respiratory virus panel (developed by Luminex Corporation, USA), which is a multiplex molecular method for the detection of respiratory viral infections. Pillet et al. 2013 reviewed elsewhere the six commercially available kits for the diagnosis of acute respiratory infections [67]. Human respiratory syncytial virus (type A and B) and FluA and FluB nucleic acids can be detected in one-step multiplex RT-PCR assay named, ProFlu-1 (developed by Prodesse, Waukesha, WI) [68].

The GeneXpert technology was developed by Cepheid, USA, and is based on the use of microfluidics and multiplexed RT-PCR for the detection and diagnosis of various pathogens. In 2010, WHO endorsed the GeneXpert *Mycobacterium tuberculosis* and rifampicin resistance assay for the diagnosis of multi-drug resistant and extensively-drug resistant tuberculosis . This platform is rapid, user-friendly, and has a clinical sensitivity of 98–100% and a specificity of 99% [69–73]. The GeneXpert platform detects and analyzes *B. anthracis* in clinical isolates within 90 min and has an LOD of 10 CFU/ml and 100% specificity [69]. Methicillin-resistant *Staphylococcus aureus* infections are also rapidly diagnosed by using the GeneXpert platform from clinical specimens [74].

After the Ebola virus disease outbreak in 2013, the GeneXpert platform was endorsed by both WHO and FDA under emergency use authorization. The assay used for the diagnosis of EBOV performed better in the field compared to other RT-PCR assays and culture methods [54, 75–77]. Real-time PCR or immunofluorescence assays are used to diagnose acute Q fever using clinical specimens (blood or formalin-fixed tissues) [78, 79]. The Primerdesign Company commercialized the Genesig Easy kit for the detection of *Coxiella burnetii* with a specificity of 66.6% and a sensitivity of 100% [80]. LightCycler real time PCR coupled with RET probes (Roche Applied Science) can be used to diagnose orthopoxvirus infection with an LOD of 5–10 copies of virus DNA in 45 min [81].

A broad-spectrum PCR coupled with electrospray ionization mass spectroscopy run on an automated platform developed by Abbott-PLEX-ID [82] is able to detect 10 bacterial and 4 viral biothreat agents listed by the National Institute of Allergy and Infectious Diseases and the U.S. Department of Agriculture, and U.S. Department of Health and Human Services [83]. The IRIDICA is a combination of PCR and electrospray ionization mass spectroscopy techniques for quick and specific detection of pathogens in patient samples. Bloodstream infections, including both bacterial and yeast, may be analyzed by the IRIDICA BAC BSI assay [66].

13.2.4 Next Generation Sequencing

Sanger and Gilbert introduced DNA sequencing in 1977. Using their method, a DNA sequence can be deciphered by adding a terminal di-deoxy nucleotide phosphates (ddNTPs) labeled with fluorescent-dye by DNA polymerase in a reaction mixture which terminates the reaction. Later, the terminated nucleic acid stretches can be detected by capillary electrophoresis, and the laser excitation values are captured on charge-coupled device (CCD) camera. The disadvantages of the system include inaccurate read out, formation of DNA secondary structures, and limitation to the short length of DNA sequences. Next-generation sequencing (NGS) opened new horizons for molecular diagnosis but still has limited use in clinical diagnosis due to the requirement of trained personnel, long duration, and sophisticated setup. This technique can be used for the detection of homopolymer or repeat sequences.

NGS is useful in the characterization of pathogen genomes, genetic mutations or drug resistance patterns, and novel pathogen discovery [84–90]. NGS application includes an unbiased in-depth analysis of complete genomes [91], whole transcriptome shotgun sequencing (WTSS) [92], whole exome sequencing and methylation sequencing, or candidate gene sequencing. Another major application of NGS includes metagenomic sequencing, which detects multiple microorganisms in homogenous or heterogeneous samples simultaneously even if microorganisms are present in low abundance. Further, NGS can be applied to non-cultivable microorganisms. The future of NGS promises great potential for the development of precision medicine. Second generation NGS includes the construction of cDNA libraries for amplification and sequencing genomes. The libraries are synthesized by fragmenting DNA strands and ligating them with adaptor molecules. Once the libraries are constructed, the adaptor-ligated sequences are amplified through emulsion PCR which uses immobilized adaptor-ligated sequences on microbeads or bridge PCR which uses solid-surface to form colonies. NGS is efficiently used for the detection of biothreat agents [93].

13.2.4.1 454 Pyrosequencing

The GS20 was the first second-generation sequencing method commercialized by 454 Life Sciences in 2005 (later acquired by Roche in 2007). This technology employs pyrosequencing chemistry in which three different enzymes, adenosine 5′ phosphosulfate (APS), luciferase, and apyrase are used. The pyrophosphates generated during the addition of nucleotides by DNA polymerases act as substrates for APS enzyme to produce adenosine triphosphate. Adenosine triphosphate is used for the conversion of luciferin to oxyluciferin by luciferase, and this conversion leads to the emission of light, which is captured by highly sensitive CCD cameras. Therefore, the amount of light produced is proportional to the number of nucleotides added to the growing chain. Apyrase degrades any unincorporated nucleotide.

The pyrosequencing system uses microscopic beads embedded with DNA sequences of 100–150 bp. The system amplifies the sequences by using emulsion PCR and provides 2,00,000 reads per run [94]. The upgraded versions of the GS20 genome sequencer, the 454 GS FLX Titanium platform, was introduced in 2007 and

provides 4–6 million reads of ≥400 bp-long sequences within 24 h [95]. The major drawbacks are the expensive reagents and inability of the system to distinguish between homopolymers (AAA or CCC), and hence the product was withdrawn from the market

13.2.4.2 Illumina Sequencing

Solexa introduced the HiSeq and MiSeq platforms for sequencing of shorter DNA templates in 2007. These techniques rely on the sequence-by-synthesis method using modified deoxyribonucleotide triphosphates labeled with fluorescent dyes for chain termination (similar to the Sanger method) and detection by a CCD camera. The 100–300 bp-long DNA ligated with adaptors is amplified on a solid surface through "flowcells" for bridge PCR amplification. The clonal amplification results in approximately more than 1 million copies of each 100–300 bp-long template. The laser excitation captures the emitted light and records the first base, the reaction is called "reversible terminator reaction". Thereafter, the cycle continues, and multiple sequence reads are recorded, aligned and compared to the reference template. Unlike 454 Pyrosequencing, which produces 1 million reads, the Hi-Seq platforms produces 120–1500 GB reads in 3–10 days, whereas the MiSeq platforms can produce up to 0.3–15 GB reads within 1–2 days for clinical testing or laboratory purposes [96]. The Illumina can detect *B. anthracis* both from the soil and aerosol samples, with a LOD of 10 genomic copies of DNA [97].

13.2.4.3 Sequencing by Oligonucleotide Ligation and Detection

Sequencing by oligonucleotide ligation and detection (SOLiD) was commercialized by Life technologies and released by Applied Biosystems in 2008. The technique is mostly similar to Roche or Illumina sequencing but differs by using DNA ligases during sequencing. After the library preparation and colony formation, sequences are detected by a modified probe named "interrogation probe." The probe is an octamer consisting of degenerate sequences covalently attached to a fluorescent dye. The first two bases are specific to the DNA template and provide 16 different combinations for annealing (e.g., AT, AG, AA, AC). The interrogation probe provides a free 5′ phosphate group instead of providing a free 3′ hydroxyl group for ligation during each step of reaction by thermostable DNA ligases. The fluorescent signals are recorded by four different channels before the last 3 bp are cleaved for the next cycle. The newly synthesized sequence is removed, and a new complementary primer binds to the n − 1 region of DNA sequence and continues the annealing and ligation cycles. Following this approach, every DNA sequence is sequenced twice, thereby providing accuracy of 99.94% to the system. The read length of this technique ranges from 25 to 35 bp-long sequences [98]. The longer duration time (7–14 days) and requirement of skilled personnel are drawbacks of this system [99, 100]. Strain-specific polymorphisms of *B. anthracis* and *Y. pestis* were studied by using SOLiD high-throughput-sequencing [101] with high genome coverage and low error rates (>99.99 accuracy).

13.2.4.4 Ion Torrent

The ion torrent platform was released in 2010 by DNA Technologies. The method uses semiconductor chips incorporated with an ion-sensitive field-effect transistor (ISFET) sensor. The ISFET is an electrical biosensor that records the changes in concentrations of H^+ or OH^- ions. ISFET sensors are used to measure the changes in the H^+ concentration after every addition of a nucleotide by DNA polymerases during a sequencing step. The biosensors convert the chemical energy to electronic signals. These ion sensors are situated right beneath microwells containing microbeads covered with amplified target molecules. The microwell chip is successively flooded with only one type of nucleotide at a time. When the nucleotide is complementary to the target template molecule at the leading position, the nucleotide will be incorporated into a growing nucleic acid. The earlier versions (2011) of ion torrents could read a 50 bp-long sequence with 99.99% accuracy and produced 100 MB of data per run. The upgraded version, the ion personal genome machine (PGM™) reads up to 400 bp with >99.1% accuracy in 7.3 h [102]. The Ion proton system, the successor to the Ion PDM, can read up to 200 bp within 2–4 h with 10 GB of data per read and can be used for genome sequencing, *de-novo* sequencing, chromatin immuno-precipitation (ChIP), transcriptome, exome, methylation patterns, gene expression by sequencing, and small RNAs [96].

NGS platforms have been integrated with several molecular diagnostic techniques which amplify even small amounts of nucleic acid randomly. This platform can detect both known and unknown pathogens in the clinical samples using the integrated digital transcriptome subtraction technique [103, 104]. The detection of a highly divergent rhabdovirus, Bas-Congo virus, through NGS highlights the potential of sequencing for the detection of novel viruses during major disease outbreaks [105]. The Ion Proton platform (BGISEQ-100) and Roche 454 v4.9 were used for sequencing and phylogenetic and phylogeographic analysis of EBOV genomes during the major outbreak in 2013–2016 [106].

13.2.5 Microarrays

Microarray technology was established in 1995, when Ron Davis and Pat Brown used cDNA as a probe to quantitate the gene expression pattern in *Arabidopsis* [107]. Microarrays are miniature lab-on-chip devices made from glass or silicon, containing 25–70 mer-long complementary oligonucleotide probes spotted on the slide through mechanical deposition [107], inkjet printing [108] or through photolithography [109]. Every single spot on a microchip contains several folds (10 nM to 100pM DNA) of oligonucleotide copies. Depending upon the need, a microarray may have multiple signature probes for different microorganisms or a complete genome from a single microorganism. The commercialized microchips from Affymetrix and Illumina have been used for the detection of more than 20,000 to several million genes. Different types of microarrays are available (protein, peptide, carbohydrate, lipid,, tissue, reverse phase, or antibody microarrays).

Steps in microarray protocols include sample preparation, labelling of probes with fluorescent dyes, hybridization of the sample on the chip, washing and image acquisition followed by data normalization, analysis and interpretation.

A highly broad-spectrum multiplexed-microarray, resequencing pathogen microarray of tropical emerging infections (TessArray® RPM-TEI 1.0, TessArae LLC, Potomac Falls, VA) detects and differentiates between 84 pathogens and 13 toxins, including Category A, B and C Pathogens with an LOD of 10^4 per test. The tests are very sensitive and can further differentiate between EBOV, Machupo virus, and Lassa virus [110].

A pan-viral array named "ViroChip" was developed for the detection of viruses. The 70-mer oligonucleotide probes recognize the conserved regions (1600 probes) from 140 viral genomes, and can be used to detect viruses, such as human herpesvirus 8, human respiratory syncytial virus, parainfluenza virus type 3, adenoviruses, and multiple rhinovirus serotypes [111]. During the 2003 SARS outbreak, a DNA microchip was used for identification and sequencing the then-uncharacterized coronavirus isolated from SARS patients [112]. An improved and upgraded version of ViroChip detects viruses of 53 families and 214 genera using full-length viral genomes [113]. In addition, ViroChip was used for the diagnosis of acute respiratory tract infection in children. Similarly, the pan-microbial array named GreeneChipPm was developed for the quick and unbiased detection of bacteria in different samples. The platform includes densely spotted oligonucleotides (29,495) from the GreeneChipVr v1.0 database. For bacterial and fungal/protozoan detection, 11,479 16s rRNAs and 18s rRNA sequences were used for pathogen surveillance and detection [114, 115].

A low-density oligonucleotide microarray can detect neurotropic viruses (meningitis and encephalitis) from cerebrospinal fluid and non-cerebrospinal fluid samples. Multiplex PCR-amplified virus sequences are hybridized to a panviral central nervous system array slide for the detection of echoviruses, human herpes virus (HHV)-2, -4, -5, -6BA,-6B, and -7, vesicular stomatitis Indiana virus, and polyomavirus JC1 [116]. These microarrays integrated with microfluidic scans detect influenza A virus [117], *Y. enterocolitica* [118], and *Bacillus* sp. in milk and various other samples. The success of microarray depends upon the sensitivity of the system to detect pathogens in a single sample even if the pathogens belong to same genus.

13.2.6 Isothermal Amplification

Isothermal amplification is a robust technique for exponential amplification of nucleic acids at a single temperature. This technique is a sequence-specific amplification without using a thermocycler, thereby cutting down the cost of instruments and making the technique easily available for POC platforms. The processing steps in isothermal amplification can be divided into three categories: (1) sequence-specific amplification, (2) enzymatic duplex melting and primer annealing, and (3) strand displacement using multiple PCR primers or strand displacement from a circular target and PCR extension with single-strand cutting.

13.2.6.1 Sequence-Specific Amplification

Sequence-specific amplification is derived from a transcription-based amplification system, which includes transcription-mediated amplification (TMA) [119], nucleic acid sequence-based amplification (NASBA) [120], self-sustained sequence replication (3SR) [121], and signal-mediated amplification of RNA technology (SMART) [122]. Unlike PCR, the primer annealing and extension occurs at a constant temperature of 37 °C, thereby reducing time and cost of equipment. This method can easily be automated and multiplexed.

These methods depend upon RNA polymerase activity for amplifying the nucleic acids (ssDNA or RNA) at an isothermal temperature, which may range from 30 °C to 70 °C depending upon the reaction. The hot-start temperature of 95 °C breaks the double-stranded DNA into ssDNA; followed by the amplification at 41 °C (or for transcription-mediated amplification at 60 °C). Amplification occurs in two phases: during the linear phase, the promoter-primer containing T7 promoter region binds at the 5′ end of the target sequence and synthesizes cDNA with the help of avian myeloblastosis virus reverse transcriptase. Subsequently, the RNA-cDNA hybrid formed is degraded by RNase H with the release of cDNA. A second forward primer binds to the cDNA containing the T7 promoter region and extends via reverse transcriptase. The newly formed strand enters the amplification phase, in which T7 polymerase binds to the DNA and synthesizes complementary RNA, again followed by the linear phase. Thus, billion-fold copies of RNA or DNA are generated within 90 min (Fig. 13.1) [123].

Signal Mediated Amplification of RNA Technology

SMART is another type of isothermal amplification. It is based on the identification and amplification of the target sequence in the sample with the help of two single stranded oligonucleotide probes (extension probe and template probe). The template probe contains a T7 polymerase promoter sequence and a transcription template. These probes hybridize together with the target RNA or DNA and form a three-way junction or "T-like" structure. The Bst DNA polymerase, which lacks 5′-3′ exonuclease activity is obtained from *Bacillus stearothermophilus*, which can amplify the nucleic acid at 70 °C. Once amplified, the target RNA or DNA is used for synthesizing the complementary strand using the extension probe. Thereafter, T7 RNA polymerase synthesizes multiple copies of RNA from the formed DNA duplex (Fig. 13.2). An enzyme-linked oligosorbent assay quantifies the SMART-generated product. Amplicons are captured and detected by biotinylated probes and separated by microfluidics or through molecular beacons [125, 126]. SMART detects genomic DNA (10 ng) and total RNA (0.1 ng) from *E. coli* in a few hours [127].

13.2.6.2 Enzymatic Duplex Melting and Primer Annealing Method

This method includes three techniques that circumvent the initial heating step for denaturing the DNA or RNA duplexes. Under *in vivo* conditions, DNA amplification is performed at an isothermal temperature, at which the DNA duplexes are opened by helicases and topoisomerases. The recombinase polymerase enzyme for amplification (RPA), helicase-dependent amplification (HDA) and rolling circle

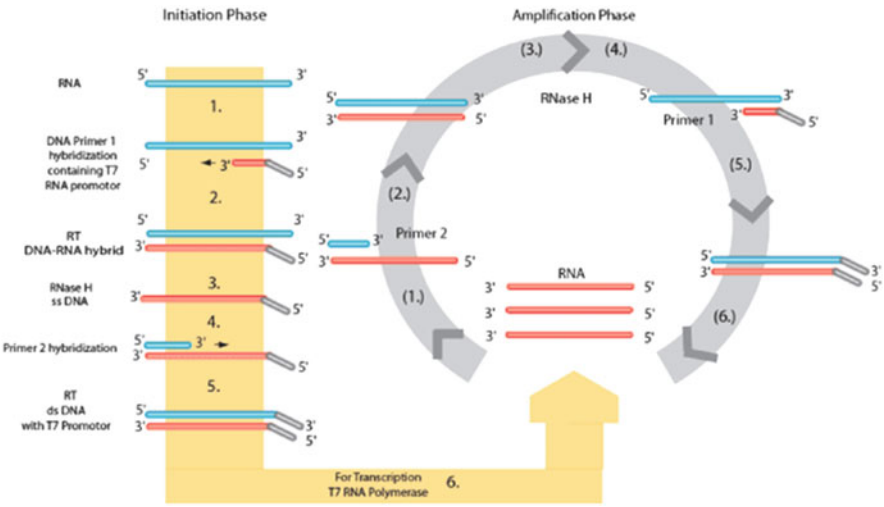

Fig. 13.1 Nucleic-acid-sequence-based Amplification (NASBA). Initial phase: 1. Annealing of DNA primer to RNA template; 2. RT for primer extension; 3. Removal of RNA by RNAse H; 4. Annealing of second set of primer to the newly synthesized DNA template; 5. Double strands of DNA are synthesized by RT; 6. For transcription, T7 RNA polymerase produces several RNA copies. The amplification phase is similar to initial phase but more copies of RNA are produced steps 1–6. (Troger V and Niemann K 2015)

amplification (RCA) enzymes amplify the nucleic acids. This technique is less time consuming, than other Isothermal amplification based diagnostic techniques, and does not require any sophisticated instruments; therefore, this method can be adopted for POC diagnostic platforms.

Recombinase Polymerase Amplification

This technique utilizes three enzymes: recombinase, single strand binding proteins (SSBs), and strand displacement DNA polymerases. Initially, the recombinase enzyme pairs with the primer and binds to the target sequence, the SSBs then bind the displaced DNA strand and form a "D" loop like structure to stabilize the DNA. This displaced DNA provides a free 3′-hydroxyl site for DNA polymerase to bind and amplify the target sequence. Therefore, both the strands are amplified exponentially within 20 min. The amplified product can be visualized either by fluorescent probes or non-fluorescent probes. RPA can be easily multiplexed by using multiple primer set and can detect more than one disease simultaneously within min (Fig. 13.3). Crimean-Congo hemorrhagic fever virus (CCHFV-AY277672 Europe 1 strain) in patient samples was detected within 35 min using isothermal amplification [128]. A commercialized RPA kit (TwistAmp) was launched by TwistDx Inc.; Cambridge, UK, for the rapid detection of pathogens, like *Y. pestis*, within 1 h with a limit of detection for ssDNA and dsDNA up to $4.04 \times 10(-13)$ and $3.14 \times 10(-16)$

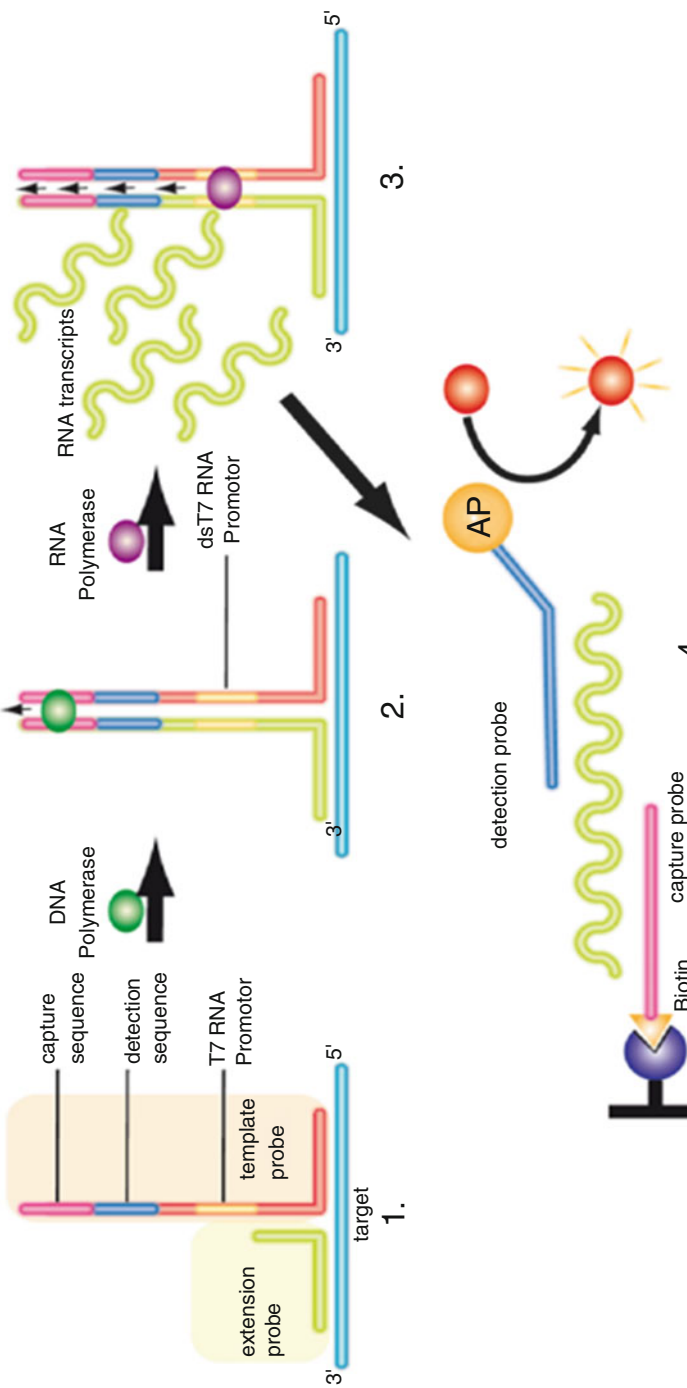

Fig. 13.2 Signal-mediated amplification of RNA technology (SMART). 1. The annealing of "extension" and "template" probes to the DNA template to form a three-way junction. 2. The extension probe is elongated by DNA polymerase, forming a double stranded T7 RNA promoter region. 3. The binding of RNA polymerase to the double strand T7 promoter produces several copies of RNA transcripts, serving as signal. 4. The signal is captured by an RNA-binding biotinylated probe, which is attached to immobilized streptavidin, and signal is detected by an alkaline phosphatase-linked probe following alkaline phosphatase substrate conversion [124]

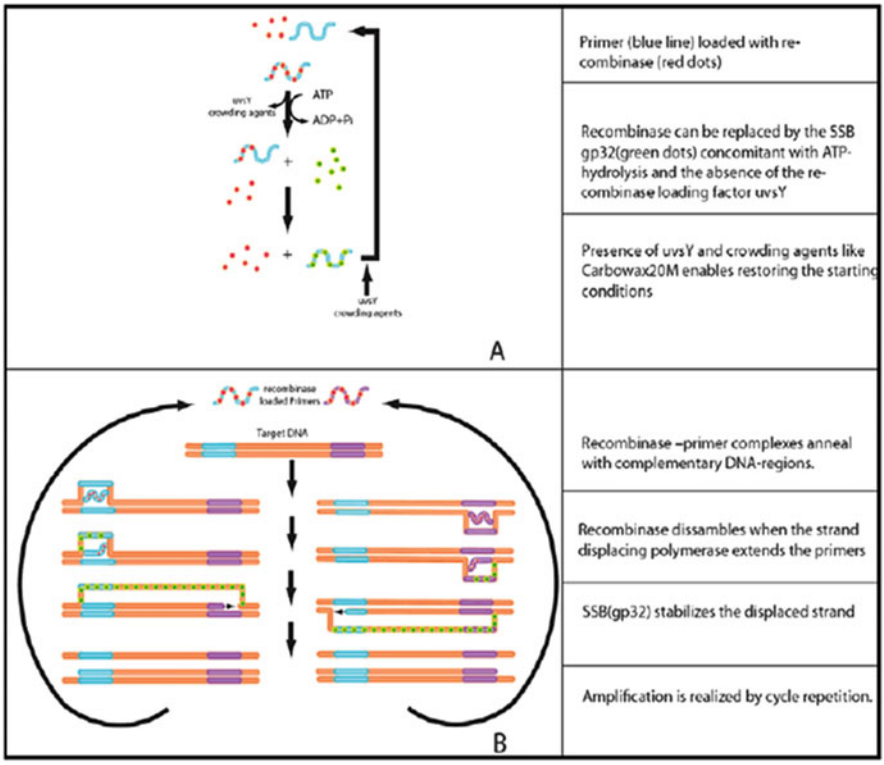

Fig. 13.3 Recombinase polymerase amplification (RPA). (**a**) The combination of the primer-recombinase complex and single-stranded- binding proteins (SSBs). (**b**) RPA cycle, in which the RPA complex anneals to the DNA template for initiation. Later, the strand displacement enzymes disassemble the recombinase and stabilize the strands by SSBs protein for further extension. Thereby, multiple copies are generated [124]

M [129]. *C. burnetii* was detected in formalin-fixed tissues and blood plasma of patients with 90% clinical sensitivity using RPA-isothermal amplification [130].

A RPA and RT-RPA fluorescent based POC platform for the detection of potentially hazardous bioterrorism pathogens (*Y. pestis*, *F. tularensis* and *B. anthracis*), gram positive and negative bacteria (*Salmonella enterica*), DNA (vaccinia and variola viruses) and RNA (EBOV, Sudan virus, and Marburg virus) was developed, with a LODs ranging from 16–21 molecules within 10 min [131].

Helicase Dependent Amplification (HDA)

Vincent et al. used DNA helicases to unwind duplex DNA or RNA and hybridize primers onto the target sequence. The enzymes separate the strands at an isothermal temperature of 37 °C, excluding the heating step [132]. The Mutl protein is a mismatch DNA repair protein found in *E. coli* that activates the DNA helicases and separates the strands. The SSBs proteins stabilize the unwinded duplex. The

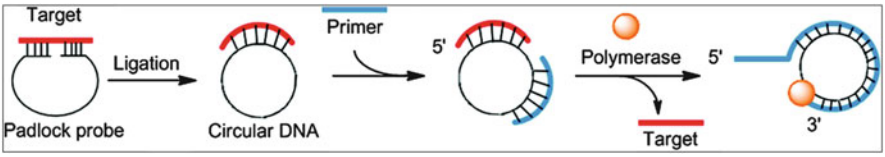

Fig. 13.4 Rolling circle amplification (RCA). The padlock probe anneals at a specific genomic location (ssDNA) forming a circular DNA and disassembles. The primers specific to the padlock probe and DNA polymerase anneal to the circular-DNA and amplify the template. These steps are performed at an isothermal temperature [124]

primers anneal to the target sequence, and DNA polymerases amplify the strands, thereby synthesizing million-fold copies of DNA within 120 min (Fig. 13.4). To further improve the sensitivity of the system, heat-stable DNA helicases derived from *Thermoanaerobacter tengcongensis* were used at 45 °C [133, 134]. The end-point results are quantified by using fluorescently-labeled probes, electrochemical detectors [135, 136], a droplet microfluidics system [137] or chip-based hybridization [138]. The HDA-TaqMan probe can detect *V. cholerae* and *B. anthracis* [139]. Motre et al. 2011 customized a HDA probe by reducing the detection time from 60 to 30 min [140].

Rolling Circle Amplification

RCA was first introduced in 1990s for amplifying the small circular DNA by using strand displacement DNA polymerases (e.g., phage phi-29 DNA polymerase of *E. coli*) [141]. RCA synthesizes ssDNA complementary to target DNA (10^5 copies from 1 DNA copy) [142, 143] at an isothermal temperature (30 °C). The method is sensitive, simple and easy to perform; therefore, this amplification method is considered as an attractive tool for POC diagnosis in clinical settings. RCA detects bacterial and viral DNA/RNA [144–146]. The amplified product can be detected by fluorescent dyes, biosensors, gel electrophoresis, electric signals, luminescence or colorimetric assays.

Padlock probes have been introduced with RCA for linear DNA amplification. The probes have two target sequences of the linear DNA at the 3′ and 5′ ends. These probes hybridize, circularize, and ligate on the target DNA and serve as a template for strand displacement DNA polymerases, simultaneously elongating and displacing the amplified product. The technique can be coupled with the set of primers that hybridize with the amplified product and synthesize hyper-branched structures (Fig. 13.5).

A liquid and solid phase hyper-branched RCA for the rapid detection of SARS-CoV RNA from clinical samples can detect a single-copy of SARS-CoV RNA in the patient sample and can be used in diagnostic setting for quick detection [147]. A colorimetric assay using hydroxy naphthol blue (HNB) coupled to hyper-branched RCA rapidly detected H5N1 influenza A virus with an LOD of 28 fM in clinical isolates. An upgrade version detects H5N1 influenza A virus using a fluorometric real time platform. The LOD of the system was 9 fM in clinical samples [148, 149].

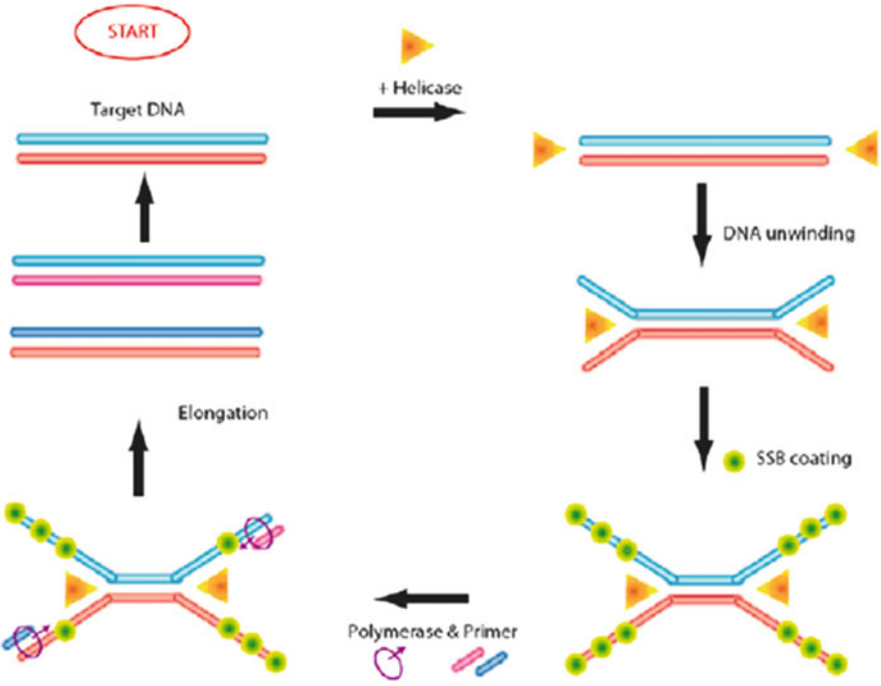

Fig. 13.5 Helicase-dependent amplification (HDA). The helicases bind to the DNA template and unwind the DNA template. The SBs stabilize the DNA strand. The primers and DNA polymerases bind to the DNA template and amplify the template [124]

Circle-to-circle amplification, another variant of RCA, detects circular DNA. Mahmoudian et al. 2008 integrated circle-to-circle amplification with a microchip electrophoresis system for the detection of *V. cholerae* with an LOD of 25 ng of bacteria within 65 min [150, 151]. An exponential linear RCA (with colorimetric detection for pathogenic bacteria, such as *E. coli*, *Salmonella* Typhimurium, and *Clostridium difficile*), was developed with LODs ranging from 10 fM to 100 fM of DNA. In this method, the amplified RCA products are cleaved by nicking endonucleases (Nb.BsrDI). The DNA fragments fold into G-quadruplex structures that form a complex with hemin and become DNAzymes. The DNAzymes undergo oxidation reactions that lead to a colorimetric detection [152].

Loop-Mediated Isothermal Amplification

Notomi et al., developed *loop-mediated isothermal amplification (LAMP)* in 2000 [153]. In this technique, the low copy number of DNA can be amplified up to 10^9 copies of target DNA within 1 h at an isothermal temperature. The basic principle is based on highly processive strand displacement DNA polymerases (*Bst* DNA polymerases) with four primer pairs (recognizing six different regions of target DNA), which amplifies the DNA and ensures the selectivity of target identification.

During the initial amplification, the four pairs of primers along with DNA polymerase amplify the DNA, forming a stem loop-like structure. The stem-loop structure becomes the template for cyclic amplification with two additional primer pairs and progresses to elongation and recycling. These additional primer pairs ensure the specificity of subsequent amplification, thereby providing a threefold amplification at every half cycle. The final amplified product consists of cocktail of stem-loop DNAs with different stem lengths and multiple loop structures (Fig. 13.6).

Apart from DNA, RNA can be amplified by LAMP using RT and *Bst* DNA polymerase [153]. Viruses, such as West Nile virus and SARS-CoV, can be detected by RT-LAMP assay [154, 155]. The amplified product is visualized by using a turbidimeter [155, 156], gel electrophoresis, fluorescent detection reagents, or by observing the production of white insoluble precipitate of pyrophosphate ions with Mg^{2+} ions [157]. The fluorescent detection reagent, calcein, and the DNA binding dye, SYBR green, used in RT-LAMP assay detect the amplified product [158, 159]. A low molecular weight cationic polymer, polyethylenimine, was used for genetic testing of West Nile virus by using Bst DNA polymerase at 63 °C [155].

LAMP is an excellent technique for POC applications. With advancement in microfluidic systems, LAMP has been successfully integrated for the specific and rapid detection of pathogens. A POC platform for the quick detection of pseudorabies virus used microLAMP (μLAMP), for on-site application with an LOD of 10 fg/μl in less than 1 h at 63 °C [160]. A similar microfluidic platform with the facility of multiplexing was developed for the detection of bacteria, with an LOD of 270 copies/μl [160]. Wang et al. 2011 designed a device that combines isothermal amplification and magnetic-beads on microfluidics chip for the detection of methicillin-resistant *S. aureus* with an LOD of 10 fg/μl within 60 min [161]. *E. coli* and *S. aureus* were detected within 30 min with LODs of 20 and 30 copies/μl, respectively, by using an electrochemical sensor made from Ruthenium hexa-amine redox molecules to detect LAMP-amplified product [162].

13.2.6.3 Strand Displacement Amplification

The strand displacement amplification (SDA) method amplifies DNA by using a multifunctional probe that has a restriction endonuclease site at the 5′ end and a strand-displacement DNA polymerase at the 3′ end. This probe extends and displaces the nicked strand. Thereafter, the displaced strand acts as a template for the second probe, leading to exponential amplification. The amplified product can be detected by fluorescently-labeled probes, molecular beacons, or intercalating dyes. SDA has been integrated with various other detection methods like gold nanoparticle-based detection on lateral flow chip or a microelectric chip-array for the detection of several mutations [163, 164]. Westin et al. 2000 developed a multiplex SDA with multiple primers immobilized on a microarray chip [165], whereas Yang et al. 2002 developed a stacked micro-laboratory platform that uses electric field for immunoassays, DNA hybridization, and amplification by SDA. These systems efficiently analyze and handle different kinds of bacteria and toxins in the sample mixture [164].

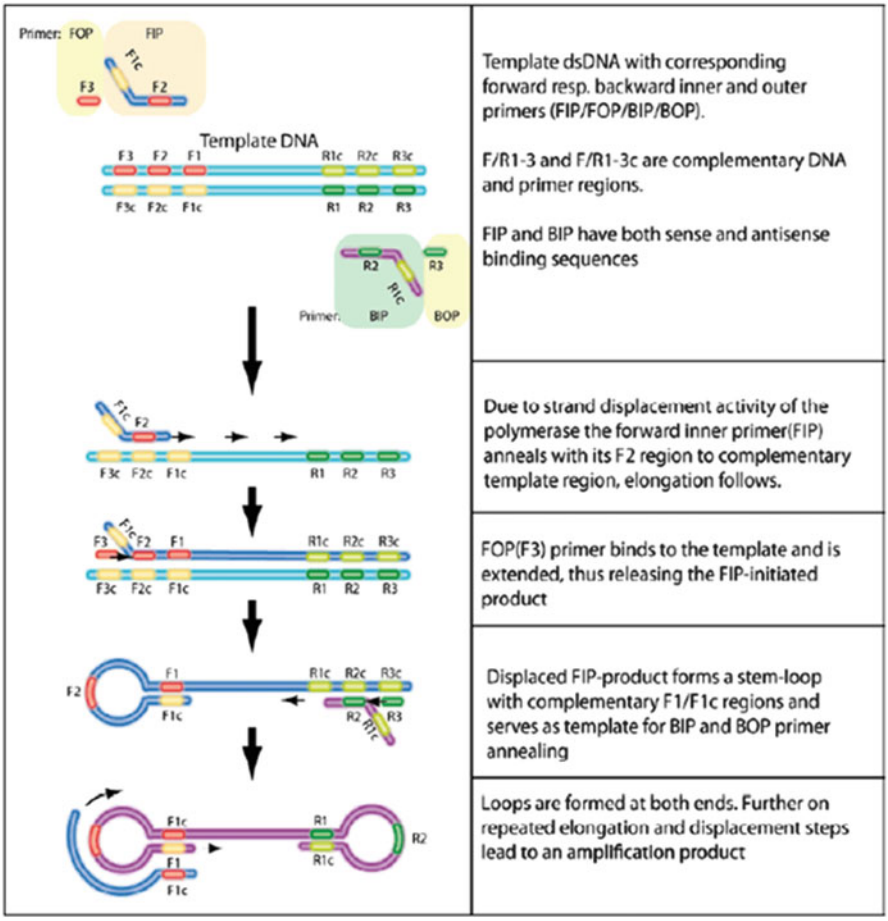

Fig. 13.6 Loop-Mediated Isothermal Amplification (LAMP). Four different primer pairs bind to different locations of the DNA template along with DNA polymerases. The Bst (highly processive strand displacement polymerase) polymerase then displaces and extends the DNA template along with the primer pair to form multiple copies of the DNA template [124]

13.2.7 Microfluidics

Microfluidics technology uses the close dimensions, preferably a channel that holds fluids with a capacity of nl to few hundred µl, for both qualitative and quantitative analysis of a given sample. The basic principle behind microfluidics technology is to create a laminar flow between the channels. The flow of fluid can be regulated by pressure-driven pumps, such as syringe pumps or electrokinetic pumps. Electrokinetic pumps apply electro-osmosis through the walls to generate pressure and flow of fluid. Microfluidics offer multiple applications in the field of molecular biology, enzyme kinetics, capillary electrophoresis, immunoassays, flowcytometry, cell manipulation, PCR amplification, DNA analysis, and clinical diagnosis [166]. The BV M-series

platform (BioVeris Corp., Gaithersburg, MD) detects antigen through electrochemiluminescence and sandwich ELISA. The antibodies, tagged with BV-TAG containing ruthenium, are immobilized on paramagnetic beads, and are passed through flow cells. The bead-antigen-TAG complexes are magnetically captured, and a voltage is applied to excite the TAG to emit multiple photons. The device is capable of detecting *E. coli* (O157), *Yersinia* sp., *S.* Typhimurium, and toxins [167, 168].

Microfluidic chips are designed by using glass, silicon or poly dimethylsiloxan, which hold small quantities of both sample and reagent in its channels for easy separation, detection and data analysis. Due to the miniaturization of the system, it is portable, cuts down the cost, and does not require any skilled labor. Therefore, microfluidics can provide a gamut of laboratory requirements on a single chip and that can be used as POC device in clinical settings.

The microfluidics platform integrated with methods such as PCR, isothermal amplification, and microarrays rapidly detects pathogens [117, 126, 137, 138, 161, 169, 170]. A lab-on-centrifugal-disk has been introduced in the field of POC devices; here, isothermal amplification (reverse transcriptase-LAMP) and optical detectors are fabricated on a centrifugal microfluidic system for the detection of influenza A virus strains. This POC device has an LOD of 10 viral copies and produces results in 47 min [171]. These lab-on-centrifugal-disk devices are more portable and robust compared to traditional assays for viral and bacterial disease diagnosis [172]. To further move in the "sample-to-result" analysis, 3M and Focus diagnostics developed a direct amplification disk that can amplify and detect pathogen in ~1 h.

13.3 Conclusion

Molecular techniques have revolutionized the diagnosis of biothreat agents and led to the development of POC devices. A number of FDA- approved and commercialized kits and devices have been launched for the rapid and quick detection of biothreat agents to strengthen the biodefense preparedness program. Although techniques like microarrays and NGS have potential to revolutionize the detection of biothreat agents, they are time-consuming and generate huge amounts of data that require skilled personnel for interpretation. Therefore, devices or platforms should be designed that are simple and user friendly and low in cost and maintenance for use in various biothreat detection surveillance programs in the field.

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Next Generation Sequencing for the Detection of Foodborne Microbial Pathogens

14

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14.1 Introduction

The rapid detection and typing of DNA belonging to known and emerging pathogens represents one of the most fundamental and frequently encountered tasks by state and national health laboratories. Over the past decade, next generation sequencing (NGS) platforms have been incorporated into a range of public health programs responsible for surveilling, detecting, and investigating/responding to infectious disease outbreaks. NGS has been rapidly integrated into the field of pathogenic foodborne microbiology as both a primary and supportive detection tool and is routinely used in the analysis of isolates from many prominent foodborne bacterial pathogens including *Salmonella*, *Listeria*, *Escherichia coli*, *Shigella*, and neurotoxigenic *Clostridium*. Collectively, 31 major foodborne pathogens are estimated to result in 9.4 million instances of illness leading to 55,961 hospitalizations and 1351 deaths per year in the United States; figures dwarfed by estimates over the same period for unspecified agents responsible for 38.4 million cases of acute gastroenteritis, 473,832 hospitalizations, and 5072 deaths [1, 2]. The relatively well-defined and studied major foodborne pathogens often are associated with established regulatory procedures for their detection and verification and are often the focus of major public health programs. A number of these bacteria have been the subject of large multi-center NGS-enabled whole genome sequencing (WGS) initiatives, which have begun to fundamentally change the landscape of disease surveillance. While a diversity of factors is responsible for the many cases of acute gastroenteritis with unspecified etiology, the possibility exists that a substantial

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number are attributable to uncharacterized, cryptic, or conditional pathogens that currently evade identification. The steady growth in WGS and metagenomic sequence data from pathogenic and non-pathogenic organisms already has provided critical insight into horizontally mobile genomic elements and revealed that some critical virulence factors may have a broader distribution than was previously understood.

NGS is a transformative technology, and the sequence data produced by NGS are impacting the field of pathogen detection in profound ways. This chapter explores what NGS platforms are, the types of sequence data they can produce, and how sequence data are being leveraged to enhance the detection of foodborne bacterial pathogens. In the first part of the chapter we begin with a brief history of the emergence of NGS technology and its early integration into the field of bacterial pathogenesis. Next, we provide an overview of core concepts used in the preparation of nucleotide data for whole genome sequencing before transitioning into an overview of several commonly encountered NGS platforms and the state of the sequence data that is produced by them.

In the second half, we focus on the utilization of NGS data as a tool for pathogen typing and detection. The process by which viruses, organisms, and/or their toxic factors drive pathogenesis can be immensely complex and diverse. Although WGS can be highly complementary to pathogen detection goals, there is no one-size-fits-all answer for how to utilize WGS data. Options will differ based on a range of factors that are often specific to the organism genome being sequenced. To provide a broad overview we discuss gnome assembly and the applications of NGS data in the context of two foodborne pathogens, *Salmonella enterica* (*S. enterica*) and *Clostridium botulinum* (*C. botulinum*). These two organisms differ substantially in regard to underlying biology, disease outbreak frequency, pathogenesis, and detection goals. These differences allow us to explore the immense variety of options an investigator faces once WGS data are acquired, highlight how these data can be applied to detection goals, and demonstrate how these goals can vary from organism to organism. We explore (1) the use of WGS as a high-resolution molecular typing tool, (2) its compatibility with other typing schemes, (3) and ways to utilize the data encoded within the genome to detect and explore virulence factors.

14.2 Next Generation Sequencing: Background and History

The most approachable use of NGS technology lies in the ability of most modern platforms to rapidly and accurately produce WGS data. For culturable bacteria, modern NGS platforms can generate WGS data suitable for de novo genome assembly or resequencing purposes within a timeframe of several hours to several days [3]. Reference-based resequencing approaches map sequenced reads to an already existing genome assembly, usually for purposes of variant detection or rapid typing as part of an established bioinformatics workflow. De novo sequencing is computationally driven, direct assembly of sequenced reads into larger contiguous

sequences. Although conceptually distinct, these applications are not mutually exclusive, and resequencing is often used following de novo assembly as part of an iterative approach to acquire a more accurate, consensus assembly. Most NGS platforms perform WGS by utilizing a random sequencing approach to generate large quantities of sequenced reads that are later computationally reassembled into a contiguous sequence, or contig. The value of this approach was demonstrated when investigators at The Institute for Genomic Research utilized random sequencing to assemble the complete bacterial genome of the pathogen *Haemophilus influenzae* in 1995 [4]. WGS data produced by most NGS platforms require a similar process of post-run assembly of sequenced reads. Pre-NGS, WGS projects relied predominantly on automated capillary sequencing or automated Sanger/ddNTP sequencing.

Though the 1990s and early 2000s, time and labor costs associated with preparing clone libraries for sequencing kept genome scale sequencing projects out of reach for most laboratory groups that were not solely dedicated to genomic projects. Most modern NGS platforms are optimized in ways that substantially simplify the library preparation steps prior to sequencing. The massively parallel nature and increased processing ability of NGS technologies enables accurate, high quality sequencing to occur in a timeframe of days or weeks, instead of the months or years required for earlier projects. Compared to automated Sanger/ddNTP sequencing, these platforms are fast, cost effective, and accessible to researchers in a range of settings. There is some sequence accuracy tradeoff, which we discuss as part of our overview of several sequencing platforms, and there can be challenges present in the storage, analysis, and effective utilization of produced data. However, the approachability of NGS has resulted in its rapid integration as an indispensable tool in the field of pathogen detection.

The first standalone next generation sequencer, the Life Sciences 454 pyrosequencer, became available in 2004. By 2008 the field consisted of a diverse collection of platforms, many operating in fundamentally different ways but all achieving the common task of producing DNA sequence data through means faster and cheaper than previous options [5, 6]. As an emerging technology, NGS adoption was not immediate. Well into the start of the NGS era, the whole genome of the *Bacillus anthracis* Ames Ancestral strain, utilized as part of the comparative genome study linked to the 'Amerithrax' investigation, was sequenced via automated capillary sequencing [7, 8]. Nonetheless, the 'Amerithax' case demonstrated the power of whole genomic data to resolve sequence differences between very closely related strains and the potential application of bacterial WGS data as an investigatory tool. Even low coverage WGS via capillary sequencing is cost prohibitive for most sequencing projects. As NGS products continue to mature as a technology, specialized pathogen oriented datasets that combine detailed isolate collection metadata, isolate WGS, and rapid phylogenetic analysis have been developed and are beginning to shape the future of pathogen detection, outbreak response, and source tracking.

While most modern sequencing platforms provide fast and accurate data, there are several important considerations when selecting a platform depending on

intended purpose. Key factors for WGS include genome size, sequencing coverage, the number of cultured isolates that require sequencing, the nature of data generated by the sequencing platform, and the availability of computing resources for assembling and interpreting the whole genomic data. Table 14.1 provides a brief overview of some of the most frequently encountered NGS platforms in use today. Each NGS platform/family of platforms determines sequence through substantially different means and prior to sequencing, extracted DNA is subjected to a library preparation step which ensures the DNA is physically structured in a way that is conducive to platform operation. Broadly, short-read sequencers such as the Illumina MiSeq, NextSeq, and MiniSeq platforms generate copious quantities of short reads that can be mapped against reference sequences or used to create de novo assemblies that assemble into multiple contigs. Long-read sequencers, including the Pacific Biosciences (PacBio; Menlo Park, California, US) and Oxford Nanopore platforms (Oxford, UK), can produce long reads for scaffolding genomic regions together and with sufficient coverage depth, produce de novo assemblies consisting of a closed genome. We discuss in detail the types of output that can be expected from the platforms covered in the table but first provide a brief introduction to some of the core concepts necessary for conducting a successful sequencing run.

14.3 Core Concepts in Sequencing

The laboratory wet work process of sequencing the genome of an organism for purposes of bacterial WGS often follows a set path. A bacterial isolate of interest is cultured on solid media and grown to desired cell density. Genomic DNA is extracted, quantified, prepared as a genomic library, and ultimately input into a sequencing platform which generates sequenced nucleotide reads as an output. Although we will describe these concepts assuming a typical WGS protocol, many are general terms that are broadly applicable to many types of sequence output.

14.3.1 Genome Size and %GC Content

Genome size, the sum of DNA in an organism measured in nucleotide basepairs (bp) varies substantially across and within the major domains of life. As of August 2017, complete genomes in NCBI/Genbank ranged in length for viruses, including viroids, from 220 to 2,473,870 bases and for prokaryotes from 112,031 to 16,000,000 bp. In the case of viral and prokaryotic genomes, genome sizes and the amounts of coding DNA increase in a mostly proportional linear fashion. On the other hand, eukaryotic genomes are extremely variable in size and often contain large amounts of non-transcribed DNA with regulatory or unknown functions [9]. For smaller eukaryotic genomes, e.g. those of some yeasts, WGS with the intention of de novo assembly may be a readily accessible but there are additional complexities by the potentially diploid+ nature of these organisms [10]. Special

Table 14.1 Sequencing statistics associated with assemblies in Fig. 14.1

Platform	# Reads	Mean Read Length (bp)	Mean coverage	Contigs	N50 (bp)	Estimated genome length (bp)	Notes
Illumina MiSeq	903,570	250 (paired, mean distance 325 bp)	40×	174	45,828	4,334,787	Preparation via Nextera XT Kit; Sequenced in a normalized run alongside 23 other bacterial samples
PacBio RS-II	40,563 reads, 142,992 subreads	11,022	250×	1	N/A	4,501,946	10-kbp size selected library

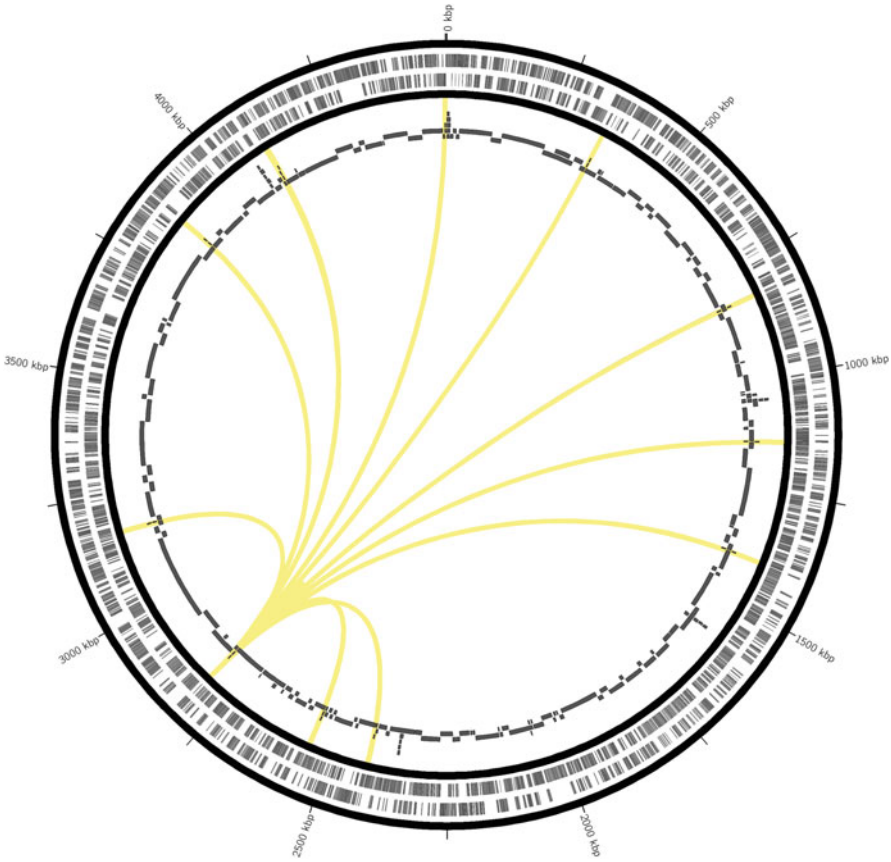

Fig. 14.1 Circos plot of a 4.5-Mbp bacterial chromosome. Solid black bands represent a closed annotated genome assembly generated from long-read sequencing on a Pacbio platform. Fragmented inner bands are sequences from a contig MiSeq short-read assembly, locally aligned against the closed genome. Yellow bands highlight the location of identical IS21 insertion sequences throughout the genome, discussed in Fig. 14.2

consideration of sequencing platform, data capacity, and bioinformatic support should be taken for projects aimed at sequencing eukaryotic pathogens such as protozoa and fungi with genomes longer than 20–25 Mbp. Figure 14.1 provides a model visualization of a closed bacterial genome with the length of 4,500,000 bp that is fairly typical of an average bacterial genome. An additional feature, %GC nucleotide content should also be considered prior to sample preparation as some sequencing platforms can have markedly different error profiles depending on the sequence composition of the loaded nucleotides. Whether empirically determined via prior sequencing or approximated from a near or distant relative organism, approximate genome length and GC% content can help to achieve the appropriate coverage necessary for acquiring informative NGS data.

Fig. 14.2 Pileup of short reads mapped against the closed reference genome showing an Insertion Sequence of the IS21 family. Blue reads represent those that map to a single genomic location, whereas yellow reads represent those that map to multiple (in this instance 10, Fig. 14.1) separate sites throughout the genome. Extended, repetitive genetic elements are often responsible for termination of contig extension in assemblies based on short reads alone

14.3.2 Coverage

In the context of WGS, coverage generally refers to the mean read depth across a given genome assembly. Calculated by the formula developed by Lander and Waterman, referred to as coverage redundancy, coverage is the product of read length by read count, over the genome size of the organism being sequenced (Eq. 14.1) [11].

$$Coverage = \frac{Read\ length\ (bp) \times Number\ of\ Reads}{Genome\ Size} \tag{14.1}$$

Greater coverage, when equitably distributed across a de novo assembly, can improve de novo assembly quality and promote more accurate variant detection in reference-based assemblies. Depending on platform read length, accuracy, and the nature of the sequenced genome itself, greater coverage can to an extent lead to more complete assemblies composed of longer and less numerous contigs. This can lead to de novo genomic assemblies that are closer representations of complete chromosomes or plasmids. Non-NGS de novo WGS assemblies tend to have coverage in the range of 5–20X, largely due to the costs associated with higher coverage. Modern NGS platforms are, by operator specification, often capable of producing coverage of bacterial genomes in the range of 10^1–10^3X. What constitutes desirable coverage is circumstantial and depends on the goals of the operator. More observations of a given site can increase the confidence of the assembly algorithm in interpreting the validity of a consensus sequence presented by the majority of overlapping reads, provided those reads are uniquely mapping, informative reads [12]. Contig extension, which is handled differently by various assembly programs, requires that the read stem from a template sequence belonging to a sufficiently unique genomic locus. This tends to be an issue with any repetitive nucleotide sequence that exceeds the length of the average read produced by the sequencing platform. Any read lacking some portion of genomic sequence flanking the repetitive sequence is uninformative and often leads to observable coverage spikes in mapped reads against the repetitive region. For purposes of sample multiplexing and efficient

use of often expensive sequencing reagents, coverage should be estimated before sequencing. Additional factors such as sample/library preparation involving a PCR amplification step can contribute to over-representation of select sequences in the sequenced reads, amplicon and shotgun-based libraries may demonstrate different error profiles [13, 14]. The operator can determine an appropriate coverage depth for their sequencing needs. The other variable of the Lander-Waterman equation, read length, tends to be much more sequencing platform-specific. We discuss read length and read accuracy in the context of specific NGS platforms.

14.3.3 Accuracy

Accuracy, the ability to correctly determine the nucleotide present at a given locus, is affected by both the accuracy of the sequencing platform and the actions of the computational assembly process on the sequenced output. In Table 14.1, we provide a brief overview of several modern sequencing platforms frequently used for bacterial WGS. Platforms differ in the types of error profiles they exhibit. Some platforms have variance in read accuracy when challenged with difficult polynucleotides, including those that are compositionally biased (e.g., high/low GC content, homopolymers, highly repetitive DNA). Others are associated with error profiles that are relatively unbiased towards nucleotide composition, but display much lower initial read accuracy and must be compensated through increased coverage. Genomic DNA library preparation is often highly prescribed; routinized protocols ensure, for instance, that the DNA sample that is ultimately input into the platform is the correct length, concentration, single/double stranded, and properly ligated with adaptors. A PCR amplification step may or may not be part of the process of preparing the sample. While beneficial in that PCR can often allow for sequencing from a smaller starting amount of DNA, PCR can also lead to the generation of PCR-induced mutations and the under/over representation of certain sequences based on nucleotide content [15]. This is more likely to be a concern during work with short-read sequencers from the Illumina and Ion torrent platforms, which often utilize a PCR step as part of the library preparation process [14, 16, 17]. Although not necessarily a problem, a PCR step may impact quality of the resulting assembly in certain circumstances. The long-read sequencers from the Pacific Biosciences and Oxford Nanopore platforms tend to have low raw-per-read accuracy relative to the short-read platforms with unbiased and biased errors respectively.

14.4 Short-Read Sequencing

A wide variety of sequencing platforms exist and many determine nucleotide sequence through fundamentally different chemical processes. One way of demarcating platforms is to consider the types of output they provide and how that output relates to the project goals of the user. Several families of sequencing

platforms, notably the Ion torrent and Illumina products, generate short sequenced reads generally in the range of ≤500 bp that tend to assemble into genomic assemblies composed of contigs. Shorter reads are often suitable for de novo assembly of small genomes, and resequencing functions when one has a closed or scaffolded genome and wants to increase coverage over certain regions or across the entire assembly. Bacterial genomes assembled from short reads alone will typically assemble into contigs. Depending on the reason why the contig ended, increased coverage may result in an assembly with fewer and longer contigs, particularly if a region simply was not sufficiently sequenced due to low coverage. But if the contig terminates because of a repetitive DNA sequence that exceeds the average read length, extra coverage depth is unlikely to result in an assembly that further integrates that contig into a longer sequence.

The Ion Torrent platforms (https://www.thermofisher.com) operate via semiconductor chips with emulsion-based, clonally amplified, bead-bound single-stranded nucleotide sequences. The chips are flooded with dNTPs in a set sequence and sensors are positioned to detect hydrogen released from the synthesis reaction that occurs when the cognate base is made available to the template-bound polymerase [18]. In Illumina platforms (https://www.illumina.com), at the start of sequencing, the sample DNA has been fragmented, flanked by adaptor sequences, and exists in single-stranded form. At the start of the sequencing run, an indexed end of the ssDNA fragment becomes bound to the flow cell. This step is followed by local bridge amplification of the DNA fragment, ultimately leading to the creation of a discrete cluster region on the flow cell consisting of many copies of the amplified fragment. The polymerase binds the complement of the adaptor not bound to the flow cell, bases containing labeled fluorophores that block the 3′ hydroxyl groups are added, excited/imaged, and cleaved of the fluorophore, regenerating the 3′ hydroxyl [3]. This process continues for a user specified and kit/platform limited number of cycles.

The applications of reference mapped and de novo genome assemblies produced by short reads are numerous. A closed genome is often unnecessary for identification of the organism at the species rank, and short-read assembles often prove suitable for more complex typing schemes. During work with an assembly derived from a pure-culture isolate, a simple nucleotide BLAST database search using a large contig is often sufficient for a match at the genus/species rank. A local BLAST query against a large curated 16S rRNA dataset such as Silva (https://www.arb-silva.de/) can also be particularly informative. A variety of approaches are potentially available, including single nucleotide polymorphism (SNP)-based trees, core genome multi-lous sequence typing (cgMLST), MLST, and k-mer-based trees. It is not uncommon during an outbreak investigation for a large number of specimens to be collected. The short-read platforms tend to have well-developed options for multiplexing large numbers of isolates on a single sequencing run, allowing for parallel sequencing of isolate's genomes and more efficient use of resources (Table 14.2).

Table 14.2 Platform specifications

Platform	Manufacture	Max read length (bp)	Max reads produced	Max output	WGS applications	Notes	Links
MiniSeq	Illumina	2× 150 bp	25 million	7.5 Gb	Viruses, bacteria, small eukaryotes/targeted sequencing	• High output kit	[1]
NextSeq 550	Illumina	2× 150 bp	800 million	100–120 Gb	Virus, bacteria, eukaryote	• High output kit	[2]
MiSeq	Illumina	2× 300 bp	44–50 million	13.5–5 Gb	Virus, bacteria, small eukaryote/targeted sequencing	• Reagent kit v3	[3]
HiSeq 2500	Illumina	2× 250 bp	4 billion	1000 Gb	Virus, bacteria, eukaryote	• High output mode	[4]
PacBio RS-II	Pacific Biosciences	Max: >60 kb Top 5% >35 kbp	0.365 million	7.6 Gb	virus, bacteria, eukaryote	• Per smart cell, up to 16 per run	[5]
Sequel	Pacific Biosciences	Max: >60 kb Top 5% >35 kbp	0.365 million	7.6 Gb	Virus, bacteria, eukaryote	• Per smart cell, up to 16 per run	[6]
Ion PGM	Ion Torrent	400 bp	4–5.5 million	1.2–2 Gb	Virus, bacteria, small eukaryote/targeted sequencing	• Ion 318 Chip v2 BC	[7]
Ion S5/S5 XL	Ion Torrent	400 bp	60–80 million	10–15 Gb	Virus, bacteria, small eukaryote/targeted sequencing	• Ion 540 chip; Ion 520 and Ion 530 Chips allow for 600 bp reads	[8]
Ion Proton	Ion Torrent	200 bp	60–800 million	10 Gb	Virus, bacteria, small eukaryote/targeted sequencing	• Ion PI chip	[9]
MinION	Oxford Nanopore	>100 kbp	Read length dependent	10–20 Gb	Virus, bacteria, small eukaryote/targeted sequencing	• Per flow cell	[10]

[1] https://www.illumina.com/products/by-type/sequencing-kits/cluster-gen-sequencing-reagents/miniseq-reagent-kit.html
[2] https://www.illumina.com/systems/sequencing-platforms/nextseq/specifications.html
[3] https://www.illumina.com/systems/sequencing-platforms/miseq/specifications.html
[4] https://www.illumina.com/systems/sequencing-platforms.html#
[5] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4678779/
[6] http://www.pacb.com/smrt-science/smrt-sequencing/read-lengths/
[7] https://tools.thermofisher.com/content/sfs/brochures/PGM-Specification-Sheet.pdf
[8] https://www.thermofisher.com/us/en/home/life-science/sequencing/next-generation-sequencing/ion-torrent-next-generation-sequencing-workflow/ion-torrent-next-generation-sequencing-run-sequence/ion-s5-ngs-targeted-sequencing/ion-s5-specifications.html
[9] https://tools.thermofisher.com/content/sfs/brochures/CO06326_Proton_Spec_Sheet_FHR.pdf
[10] https://nanoporetech.com/products/minion

14.5 Long-Read Sequencing

One of the drawbacks of short-read sequencing is that de novo assemblies utilizing only short reads rarely assembly into a closed genome for most bacterial and eukaryotic organisms. Closed genome assemblies from short-read data often rely on a reference or additional targeted sequencing methods. The primary reason for this drawback is that if a repetitive region of DNA exceeds the average read length produced by the sequencer, a large quantity of reads will appear identical and the computational assembly program is unable to appropriately place them in the assembly.

There are many situations in which a closed genome is highly desirable for investigating the pathogenicity of an organism. For instance, many virulence factors are horizontally trafficked on composite transposable elements. For instance, it may be of interest to examine the full length of that mobile sequence for unique virulence factors, to determine whether it is on plasmid or chromosomal sequence, or exploring flanking genes for comparative genome analysis. Although increasing coverage depth may be sufficient to ensure the entirety of the sequence assemblies on a single contig, if it is composed of largely non-redundant nucleotides, longer reads are necessary to overcome longer repeat regions. Mobile genetic elements can operate in ways that substantially increase nucleotide sequence redundancy across a genome. Bacterial ISs often fall in the range of 1–2 kbp in length, which is in excess of the average read length produced by most non-paired end short-read sequencers [19]. Some have a copy-paste duplication feature that enables further propagation of the redundant sequence throughout the genome [20]. Through exploitation of endogenous and transferred homologous DNA repair processes, insertional and chromosomal material can be duplicated and altered in numerous ways [21]. As illustrated by Fig. 14.1, examination of the ends of contigs generated from de novo assembly of MiSeq 250-bp paired reads often align with annotated insertional sequence transposase coding sequences that are present at many different sites throughout the bacterial genome. Several sequencing platforms produce reads that average in the range of 5–100 kbp, which is often sufficient to overcome repetitive sequences and produce complete bacterial genomes.

The PacBio RS-II and Sequel platforms can produce a wide range of read lengths depending on user library preparation, with the longest 5% exceeding 35 kbp. As a result, the platform is quite attractive for generating closed bacterial genomes and plasmids. The raw reads have a fairly high single pass error rate of 11–15%, which consists primarily of indel type errors [22, 23]. Each base would have roughly an 11–15% chance of being erroneous if one were only evaluating a single-sequenced read produced by the polymerase. These errors are reported to be unbiased in regards to the nucleotide content. The circular nature of *SMRT-bell* library enables multiple sequencing passes, allowing for generation of subreads that can be utilized for determination of the consensus nucleotide at a given site during genome assembly [22]. In concert with the data acquired from parallel sequencing reactions

overlapping the same site(s), which further contribute to coverage depth, final assemblies attain accuracy in excess of 99.999% [22, 24]. Figure 14.2 shows a close up of MiSeq short reads mapped to the PacBio consensus sequence of Fig. 14.1. The yellow pileup represents non-specific reads that map to the template in multiple locations across the genome. This particular pair of genes encodes an IS21 insertion sequence, which reoccurs seven times with 100% nucleotide identity throughout the genome. Such features often result in contig termination or misassembly in short-read-only assemblies that lack a reference sequence. Long-read sequences are often critical to studies seeking to perform the highest resolution typing possible between closely related bacteria of the same species and those studying broad genomic arrangement.

Although all previously discussed platforms have operated in distinct ways, they all utilize the principle of sequencing by synthesis. The DNA sample of interest is prepared in a way conducive to platform operation, and the platform determines the sequence by synthesizing a complement to the input DNA. The relatively new Oxford Nanopore MinIon platform instead operates by utilizing biological pores and measuring changes in conformation associated with each nucleotide that passes through them, in effect bypassing the need for synthesis on of the input molecule [25]. Error rates in sequenced reads are in the range of 12–35% during a 2-dimensional (2D) double pass sequencing run [26, 27]. MinION reference consortium data from 2017 utilizing the new R9.0 chemistry had a total 2D error rate in reads of 7.5% and were associated with bias towards reads with greater GC content as the run progressed [28]. A reported de novo assembly of a 4.6-Mbp *Escherichia coli* genome with 30× coverage achieved an accuracy of 99.4% [29]. Low accuracy and a developing bioinformatic suite has so far mostly relegated MinION use to reference mapping or the scaffolding of contigs produced by other platforms. This is likely to change as the platform becomes better established and the chemistry continues to be updated. MinION determines base identity as a nucleotide passes through a nanopore and past changes to the pore have had significant impacts on accuracy [28, 30].

The low cost, rapid library preparation, real-time accessible, long reads produced by the MinIon make it a unique addition to the existing field of NGS platforms. In one study, the longer reads were used to scaffold across a repeat region in *Salmonella typhimurium* unable to be resolved using short-read data alone, in turn enabling investigation of an antibiotic resistance island [31]. A hospital-based study tracking a *Salmonella* disease outbreak in multiple patients, using cultured sample derived from patient stool, was able to acquire sufficient data to identify the sample as a *Salmonella* sp. in 20 min, and as serovar Enteritidis within 40 min of sequencing [32]. Several studies utilizing Illumina platform short reads to polish accuracy coupled with long-read scaffolding produced by the MinION were able to assemble a closed bacterial chromosome and a variety of closed plasmids [33, 34].

14.6 Using Next Generation Sequencing Data for Pathogen Detection

Short-read sequencing is generally the starting point for most bacterial sequencing projects. Short-read sequencing platforms tend to allow for the multiplexing of large numbers of isolates at coverage levels acceptable for de novo assembly or read mapping within a single run. Short reads have generally proven more cost effective than their long-read counterparts in terms of both capital and reagent cost. De novo assemblies built from WGS data produced by short-read sequencers are sufficient for purposes of gross identification at the genus/species rank, gene annotation, and investigation of gene products, and can be subjected to a number of traditional and NGS enabled subtyping methods. Long-read sequencers can produce output that can assemble into a closed genome. A closed genome can be used to enable high resolution typing by serving as a reference for very closely related isolates, and allow for the investigation a variety of genomic features and organizations including many associated with pathogenesis and horizontal gene transfer.

This is particularly the case when the underlying genomic diversity in bacteria of a given species is vast or unknown. Botulinum neurotoxin (BoNT), the causative agent of foodborne botulism, is the most potent known biological toxin as estimated by its LD_{50} [35]. BoNT is produced by several species of clostridia including the polyphyletic *C. botulinum* from the *bont* gene, which generally occurs within horizontally trafficked gene clusters roughly 15 kbp in length [36]. Despite most *bont* gene clusters sharing a conserved gene organization/synteny, they often lack broad nucleotide identity. At least eight antigenically distinct BoNT serotypes have been identified and hybrid toxins derived from recombination between serotypes also exist; amino acid identity across serotypes can be as low as ~30% [37–40]. The *bont* gene clusters can be part of chromosomes, plasmids, and phages; bi/tri-toxin producing strains of *C. botulinum* containing multiple *bont* clusters within the same genome have been reported [41]. The relatively limited availability of complete genomes (25 in GenBank as of August 2017), rarity of outbreaks, and shear number of potential combinations of species/strain and toxin serotypes, would generally favor de novo assembly over read mapping when evaluating a newly sequenced isolate or strain genome. Short-read sequencing is generally a strong starting point and with sufficient coverage may result in the inclusion of the entire toxin cluster in a single contig. Long-read sequencing might also be beneficial to ensure the toxin cluster(s) are complete, and can be analyzed with less ambiguity as to their genomic contexts. Additionally, horizontally mobile elements often co-occur at the flanks of the toxin clusters and can frequently terminate contig extension during assembly or result in misassembly. The pathogen genome being sequenced can have unique implications for sequencing, but a de novo assembly produced from short reads is often a strong place to start.

If a wealth of high quality reference genomes already exist, short-read data can be sufficient to infer important genomic organizations with high confidence based on read mapping against a reference sequence. Read mapping is routinely utilized to determine the presence or absence of certain genes and genomic regions reads from

S. enterica relative to reference strains and plasmids [42, 43]. The read-mapped consensus sequence, in the absence of large quantities of unmapped reads and large coverage gaps, can be used to generate a SNP matrix from variant SNPs between the mapped isolate short reads and the closed reference genome. The SNP matrix can be used to calculate distance to generate a phylogeny. Creating a SNP matrix is a complex process and is generally only desirable in situations during which 10s–100s of closely related isolates differ from a closed reference genome at only several hundred SNPs [44]. The resolution provided between strains at this level is unrivaled and allows for extremely detailed subtyping between highly clonal isolates. To explore the relationship between 47 isolates of *Listeria monocytogenes* derived from clinical and food samples in association with a listeriosis outbreak and their relationship to several isolates from a separate outbreak that appeared identical by Pulsed-Field Gel Electrophoresis (PFGE), Chen and colleagues determined closed genome sequences for a pair of isolates to serve as high-quality references and to clarify several putative prophage regions. A SNP-based distance matrix was able to discriminate between the two outbreak strains [45]. When integrated into a database containing a wealth of sequenced clinical, food, and environmental isolate genomes and collection metadata, the subtyping resolution provided by NGS revolutionized outbreak response and outbreak detection. Currently this approach is mostly limited to several heavily sequenced pathogen genomes. However, increasing access to NGS technology, the emergence of a low cost long-read sequencer, and the immense growth in publicly available sequence data may soon make high-resolution subtyping available and attractive to a broader range of investigators.

Bacterial genomes are haploid and should be invariant in regard to nucleotide composition at a given site if the sample was derived from pure culture. Novel mutations may arise during the culturing process and each platform has its own quirks regarding raw read accuracy. Assuming the absence of sample contamination or extremely low coverage, these features are unlikely to interfere with generation of an assembly sufficient for gross identification at the genus/species rank. For certain WGS detection goals, such as the precision subtyping of isolates of the same species through a SNP matrix, it is important to have an awareness of how sample preparation and platform selection can impact accuracy. Short reads can be useful, even when the research goals might strongly suggest the value of obtaining a closed genome. Short-read sequences can be used to reduce, or alter, the error profile present in the closed genome sequence(s). The SPAdes genome assembler (http://cab.spbu.ru/software/spades/) allows hybrid assemblies that can utilize both long and short reads from the platforms surveyed above [46], whereas the program Pilon can use high coverage short reads from a short-read sequencer to detect and correct variants within larger contigs, scaffolded assemblies, or closed bacterial genomes [47].

In terms of software for de novo whole genome assembly, investigators are presented with a wealth of options. We will not delve too deeply in to this topic, but we provide a brief overview of options. Many NGS platforms offer packages that provide options for accessing an in-house or affiliated assembly suite for use with the data created by their sequencers. Consolidated commercial assembly suites such as CLC genomics workbench (QIAGEN Bioinformatics, Denmark) and

Geneious (Biomatters, New Zealand) provide support for a wide range of read types. Additionally, an extremely wide variety of freely available assembly programs, such as Velvet and SPAdes, are available [46, 48]. Of the last category, several options provide graphic user interfaces, but many require use of a command-line and Unix shell. Depending on intended application, it may be of additional benefit to review the manual and/or documentation of the assembly program being used. Assembly programs often operate using either an overlap-layout-consensus or de-bruijn-graph based algorithm which can impact computational performance and assembly quality [49]. While beyond the scope of our review, how the assembly program interacts with factors including the genomic makeup of the sequenced organism and the sequencing platform can significantly impact the completeness and accuracy of the final assembled genome.

Over the past decade, NGS has mostly played a supportive role in pathogen detection. NGS is frequently performed on a microbial culture that has already been selectively isolated and uses either as an additional confirmatory test alongside other tests [e.g., metabolic tests, primer specific polymerase chain reaction (PCR)], or for typing purposes after the identity has been largely confirmed by other means. We will explore how NGS has been integrated into existing typing schemes using Non-typhoidal salmonellae, the leading cause of bacterial foodborne illness, as an illustrative example.

14.7 Applications of Sequencing Data for Pathogen Detection

14.7.1 *Salmonella enterica* in a Public Health Context

Non-typhoidal *Salmonella* are estimated to be responsible for one million annual cases of foodborne illness in the United States and are the leading cause of hospitalization amongst 31 foodborne pathogens [2]. As highly prevalent bacterial pathogens associated with significant morbidity, salmonellae's historic and continuing importance as a pathogen avails the investigator access to a wealth of data concerning the detection, identification, and epidemiological typing of *Salmonella*. In a case of suspected foodborne *Salmonella*, well established, selective isolation protocols from a wide variety of foods are readily available, as are serological tests, metabolic tests, and visual guides for identifying typical/atypical colony morphology [50]. These methods for isolation and confirmation are well established and validated. Despite having undergone revisions over time, changes tend to be incremental resulting in a set of stable, streamlined, and regulatory compliant methods that are accessible by local and clinical health authorities. Although these procedures reliably provide genus confirmation, the selective isolation process alone is generally insufficient for detailed further typing at the species/subspecies ranks. Concerted efforts to surveille *Salmonella* to investigate and prevent their entry into the food supply have been ongoing for 70 years. The ability to categorize and differentiate *Salmonella* strains is central to an effective surveillance program and a multitude of salmonella typing schemes have been used in pursuit of the best means of differentiating between strains (Fig. 14.3).

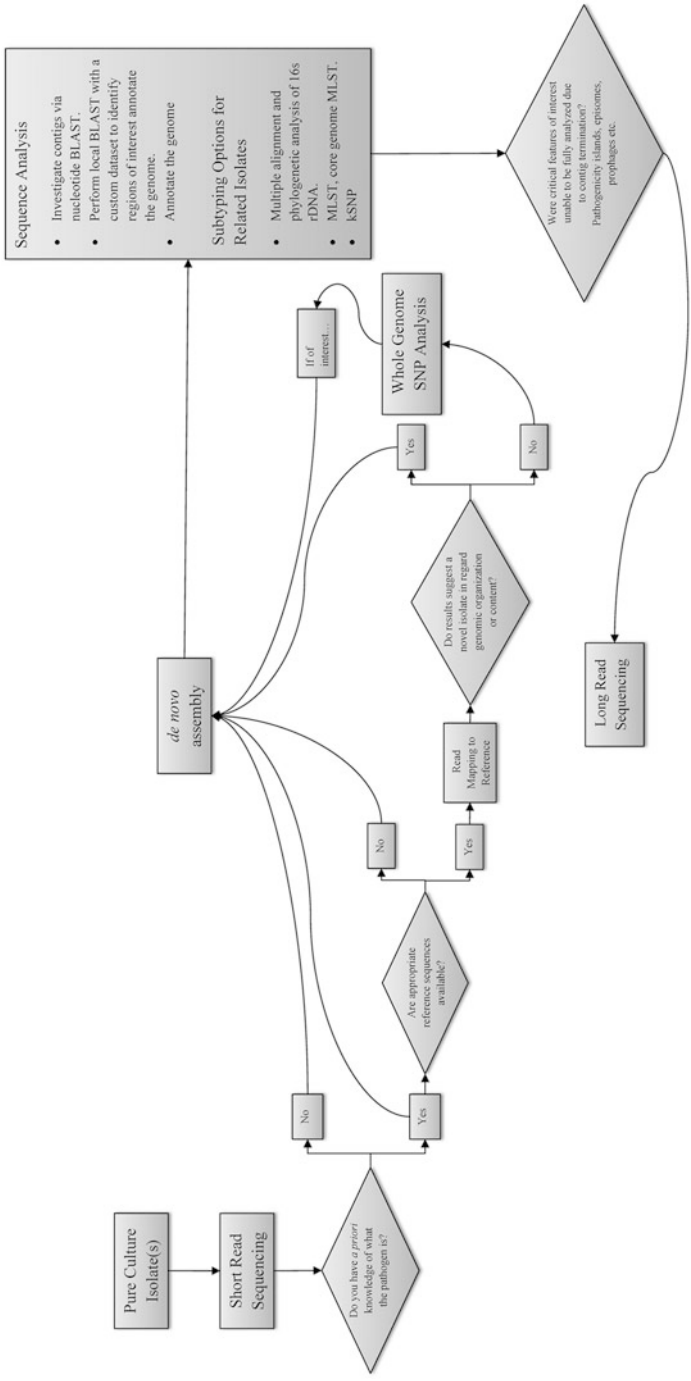

Fig. 14.3 A generalized sequencing scheme for a bacterial pathogen

14.7.2 WGS and Specialized Epidemiological Databases Enable Pathogen Typing at Unparalleled Resolution

Outbreak response is bolstered substantially through the coupling of surveillance metadata with accurately, and specifically typed outbreak isolates. In the U.S., national surveillance efforts for salmonellae were first established in 1963 [51]. Initial surveillance provided valuable insight into the complex ecology of *Salmonella* as pathogens within the food supply ranging from improved understanding of what constituted high risk foods, the impact of mechanized processing activities, and the potential utility of exploiting biochemical differences between strains, trace contaminated foods back to their probable source [52–54]. Utilizing serotyping data, surveillance efforts in the 1960s–1980s were occasionally able to trace food back to their probable source across international borders, though most source tracking successes from this time period occurred at the national level [55, 56]. The increasing globalization of the food supply chain coincided with an increase in the frequency and scale of foodborne salmonellosis outbreaks [57]. Greater diversity in the supply chain and increased opportunities for exchanging products and pathogens placed further emphasis on the importance of being able to discriminate between salmonellosis outbreak strains.

14.7.3 Salmonella Serotyping and Inferring Salmonella Serotyping via PCR

Salmonella classification/typing by serotype under the White-Kauffmann-Le Minor scheme organizes *Salmonella* strains by surface antigen [58–60]. The method predates DNA based molecular methods, remains in practice today, and illustrates how advances in molecular typing methodologies can build upon one another. As of the last major WHO update in 2007, 2557 *S. enterica* serovars were described based on a combination of O, H, and Vi antigens [59]. The subtyping process takes several days, is labor intensive, and often proves impractical to perform on more than a small fraction of individual isolates associated with a given disease outbreak.

A variety of approaches, including specialized high-throughput mechanical/automated serotyping, PCR-based methods, and most recently NGS have been used to decrease the time and labor necessary to determine serotype [61–63]. Allele-specific PCR methods were developed that target the genes encoding the antigenic determinants enabling inference of serotypes from analysis of PCR amplicon band patterns on electrophoretic gel. Such methods are reported to have reduced the time from start to finish to 5 h with high concordance between physical typing and the PCR method (108 of 111 tested isolates in one such study) [61]. Comprised of the entire genomic nucleotide content of a given organism or even a given cell, WGS data provides unparalleled insight into the gene content, organization, and predicted coding sequences present. Most allelic typing schemes are or can be made compatible with WGS data.

14.7.4 Inferring Salmonella Serotype via WGS Assembly

The Seroseq webserver accepts WGS reads in fastq file format and assigns *Salmonella* serotypes by searching for the specific alleles associated with the *fliC*, *fljB*, and *rfb* genes that encode the serovar-determinative proteins [63]. NGS allows the preparation and multiplexed whole genome sequencing of 100s of individual *Salmonella* isolates at once. Although the preparation time involved for WGS on a short-read sequencer with high capacity for multiplexing, such as the Illumina benchtop platform family, for 100–200 samples is generally several days, it can be conducted by a single individual using a streamlined process that does not require the use of primer-specific PCR in conjunction with gel electrophoresis to visualize results. With WGS involved as a routine part of outbreak response, this approach allows an assessment of serotype from many samples that would not have been subjected to serological analysis. This inclusion also enables generation of trees that reflect antigenic, rather than phylogenetic ancestral traits, which enable tracking horizontally mobilized genes including those that confer antibiotic resistant and virulence traits.

14.7.5 NGS Data Can Often Be Integrated with Existing Allelic PCR Based Typing Schemes

Most short-read assemblies of salmonella isolates are equally amenable to MLST, cgMLST, and any sort of allele-based typing scheme that the user desires. Some have argued for the replacement of *Salmonella* serotyping with an MLST scheme composed of allelic variants of several well conserved housekeeping genes that together provide a level of discriminatory capacity. This method is generally more consistent with phylogenetic trends observed when using higher resolution methods [64]. The value of WGS data is that they allow the investigation of both the phylogenetic ancestry of the organism and the phylogeny of any subset of nucleic acids common to the isolates being investigated. The National Antimicrobial Resistance Monitoring System (NARMS) characterizes and monitors spread of antimicrobial resistance (AMR) genes. Investigators were able to compare rapidly the antimicrobial resistance profile of 640 *Salmonella* isolates by searching against several AMR databases with 90% correlation between genotype and in-vitro tested phenotype [65]. Specialized datasets such as those specializing in AMR grow in value as more functionally characterized genes/protein-encoding sequences are entered into them. In such a setting, discordance between phenotype and genotype can lead to novel discoveries and flag flaws in predictive workflows. Furthermore, this setting enables the utilization of sequence data in conjunction with associated gene products and other publicly available sequence data that can provide critical insight into pathogenesis at the molecular level.

14.7.6 WGS Is an Unparalleled Tool for High Resolution Bacterial Subtyping

Although collection of metadata is an invaluable tool in source tracking, it is only as useful as the resolving power of the typing method used. Pulsed-Field Gel Electrophoresis (PFGE), a method developed to separate large nucleic acids, typically utilizes a restriction digest that generates a unique pattern on an electrophoretic gel and often comparison of the pattern is sufficient to distinguish between different *Salmonella* strains [60]. In the U.S., PFGE has been heavily utilized for *Salmonella* typing as part of the PulseNet surveillance network and within 5 years of operation, PulseNet USA contained 110,000 PFGE *Salmonella* profiles [66, 67]. Although generally able to discriminate between *Salmonella* strains, PFGE can be insufficient when very closely related strains are analyzed and lacks the resolution necessary to indicate differences between isolates from the same outbreak [68, 69]. To further bolster its discriminatory capacity, PulseNet incorporated additional amplicon based methods for *Salmonella*. One such typing scheme, multi-locus variable number tandem repeat analysis (MLVA), is PCR = based and utilizes predesigned primers that produce amplicons that vary in size if variation in repeat length is present across strains. Due to the high concordance between MLVA and PFGE results and the increased resolution of MLVA over PFGE, MLVA may be used following PFGE to increase resolution between visually non-discernable strain pulsed-gel profiles [69].

NGS both bolsters and disrupts previous typing schemes for salmonellae. NGS enables the rapid whole genome sequencing (WGS) of *Salmonella*, and virtually any culturable bacterial organism. One can directly compare two or more *Salmonella* strains/isolates across all available sites, and this is readily achieved through a whole genome SNP analysis using high quality reference strains. For typing purposes, the resolution of WGS data alone is theoretically equal to the total nucleotide count present in the genome assembly, including the chromosome and the sum of any present plasmids. *Salmonella* genomes typically range in length from 4.4 to 5.8 Mbp [70]. The data produced through cross-genome analysis of WGS data allow for greater resolution than previously discussed methods, and can differentiate between *Salmonella* strains that cannot be distinguished by PFGE. An early application of a WGS as a subtyping tool was demonstrated through its ability to distinguish *Salmonella* Montevideo isolates, which generally appear identical in PFGE. WGS of 47 strains of *Salmonella* Montevideo led to the identification of 23 informative SNPs that concurred with their respective disease outbreaks upon analysis within a derived phylogenetic tree [68].

As of 2018, 5 years after its creation, the GenomeTrakr database contained just over 105,000 genomes of *S. enterica* isolates. Government and academic laboratories now routinely use WGS as part of their pathogen detection workflows and the scale of this cooperation and the accessibility of the data continue to grow. The pathogen detection (https://www.ncbi.nlm.nih.gov/pathogens/) data are further enhanced by a wealth of metadata that enables rapid outbreak response and new avenues for outbreak prevention [71]. Greater resolution allows for more precise matching between environmental, food, and clinical samples.

14.7.7 WGS, Public Databases, and Bioinformatics Enable New Means for Discovering Putative Virulence Factors

Returning to the example of foodborne botulism, the preformed BoNT is the causative agent and detection protocols generally focus on identifying the BoNT serotype as it is often the primary indicator of disease phenotype, duration and progress [72]. Contrary to salmonellosis, foodborne botulism detection methods generally prioritize toxin detection and serological typing first, with a secondary emphasis on isolating and growing the organism. WGS has provided insight into the horizontal transfer, recombination, and evolution of BoNTs. Over the past decade alone, NGS has led to the discovery of new hybrid toxins, silent toxins, novel serotypes, and the discovery of BoNT-like toxin homologs in organisms beyond members of the genus *Clostridium*. In doing so, NGS has forced a spirited debate over the very definition of a BoNT serotype and demonstrated how the targeted sequencing of known pathogens supports this work.

14.7.8 Role of WGS in Investigating a Hybrid Botulinum Toxin Consisting of Two Serotypes

In 2013, investigators described a novel botulinum toxin produced by a bivalent Group I *C. botulinum* strain isolated from infant stool in a case of infant botulism. Initial efforts to serologically type the toxins using Centers for Disease Control and Prevention (CDC)-provided monovalent polyclonal antibodies for known serotypes A–G resulted in identification of a B serotype toxin and an unknown toxin non-neutralized by the antibodies referred to as BoNT/H [37]. WGS of the clostridium strain revealed that the coding sequence for the unknown BoNT serotype had a mosaic/hybrid composition similar to that of BoNT/F and BoNT/A serotypes. Subsequent investigation indicated the BoNT/H type toxin can be neutralized with BoNT/A antitoxin [38, 39]. Debate persists over nomenclature, the use of research vs. non-research antitoxins in achieving neutralization, antitoxin dosage and potency, and whether the BoNT/FA(H) hybrid represents a novel serotype in its own right [73]. However, determination of the BoNT/FA(H) hybrid nature via WGS meaningfully informed neutralization research that could augment treatment of BoNT/FA(H) intoxination or intoxination by closely related homologs should they emerge again in the future.

BoNT's are significantly diverse across serotypes at the primary amino acid level but substantially conserved in regard to core motifs and domains. Naturally occurring recombinants such as BoNT FA(H) demonstrate that hybrid serotypes can be capable of causing disease. Some serotypes, including BoNT/C, BoNT/D, and BoNT/G, have rarely, if ever been associated with a human case of botulism, though BoNT/C/D and their hybrids are major sources of botulism in wild animals and livestock [74–77]. WGS has revealed that such multivalent clostridium strains such as that producing BoNT/FA(H) and BoNT/B are not particularly unusual, and B serotype clusters with nonsense mutations have also been observed in a number of

isolates [78]. While not human disease causing, toxin fragments and non-implicated serotypes may remain relevant through their capacity to recombine with serotypes more frequently responsible for botulism outbreaks. Heptavalent botulinum antitoxin (HBAT), an equine polyclonal treatment against BoNT serotypes A–G, is the primary treatment for foodborne botulism in the US [79]. Assuming hybrid toxins remain antigenically similar enough to their constituent serotypes, existing polyclonal antitoxin treatments should have the capacity to bind novel hybrid types. HBAT is effective against BoNT/FA(H) [80]. However, WGS has also revealed that additional antigenically distinct serotypes exist. Bioinformatics analysis of WGS data revealed in 2017 the first novel BoNT serotype discovered since 1970 [40, 81].

14.7.9 Discovery of a Novel Botulinum Neurotoxin Serotype via Nucleotide/Protein Databases

A novel serotype, BoNT/X was recently described and represents the first to be identified through bioinformatics. *C. botulinum* strain 111 was initially isolated from a case of infant botulism in 1996 and was observed, at the time, to test positive for BoNT/B [40, 82]. A closed genome assembly of *C. botulinum* strain 111 was released publically by NCBI in 2015 with a plasmid-borne BoNT/B2 toxin gene cluster and a previously undiscovered chromosomal BoNT toxin gene cluster containing a putative BoNT with low identity but broad homology to all known serotypes [AP014696.1]. Experiments utilizing partial and sortase linked recombinant BoNT/X found that it cleaves several traditional and non-traditional SNARE substrates, is antigenically distinct from known serotypes, and causes flaccid paralysis in laboratory mice [40]. It remains unknown whether BoNT/X is expressed by *C. botulinum* strain 111. The ongoing investigation into BoNT/X highlights how bioinformatics-driven investigation of WGS data can allow detection and subsequent analysis of cryptic virulence factors that may not be detectable through other means. BoNT/X evaded detection in the 1990s and was captured through WGS conducted 20 years later. Within 2 years of the sequence being made public, researchers had bioinformatically characterized, artificially synthesized, recombinantly expressed, and demonstrated the enzymatic functionality of a novel BoNT serotype.

14.7.10 Identification of Botulinum Neurotoxin-Like Proteins in Bacteria Beyond Genus *Clostridium*

Interestingly, both the BoNT FA(H) and BoNT X toxins were discovered in dual toxin-producing strains of *C. botulinum*. The *bont* gene cluster exists within flanking ISs, prophages, and plasmids suggesting some degree of horizontal mobility. A complete toxin cluster encoding an enzymatically functional botulinum toxin was recently identified on a plasmid of an *Enterococcus faecium* isolate that was obtained from cattle feces [83]. Like BoNT/X, this cryptic toxin was identified through bioinformatics driven investigation of sequence databases. Even more

divergent BoNT-like-encoding sequences with broad homology to those encoding BoNT have been identified in a range of bacteria [84–86]. Although some of these organisms, including *Enterococcus* and *Weissella*, share a similar ecological niche as *Clostridium* and require similar strict anaerobic conditions for growth, others do not. The sudden marked growth in the apparent prevalence and diversity of botulinum toxin homologs is unlikely to be an isolated occurrence. The increased diversity and depth of sequence databases is revealing that many important virulence factors may have similarly complex horizontal distributions.

Understanding the horizontal transfer of critical virulence factors, coupled with a large global database of sequence data, can provide new insight into virulence factor evolution and potentially flag emerging or previously unknown pathogens for further investigation. In the case of botulinum neurotoxin, this coupling has led to the discovery of distant toxin homologs in bacteria of unrelated species. WGS opens new avenues of cross-species investigation into horizontally transmitted genes and gene products that include many fundamental molecular building blocks that enable bacterial pathogens to cause disease. WGS allows not only the investigation and comparative analysis of the nucleotides present within the sequenced isolate genome, but also the investigation of its gene products, and the utilization of any associated metadata. The integration of these data in the fields of outbreak detection, response, and prevention have enabled the development of robust public health programs.

14.8 Conclusions

NGS has become a technology central to pathogen detection and characterization. WGS is a useful tool for high resolution typing of closely and distantly related bacterial pathogens, and has already changed the landscape of disease surveillance. In contrast to the previous high-resolution typing standard, PFGE, WGS also produces an abundance of data at the gene/allele level that can provide in-silico backwards compatibility with PCR-based typing methods such as MLST. For closely related isolates for which suitable reference sequences exist, a variety of SNP-matrix based typing schemes can be used and for those lacking such reference sequences, a cgMLST approach can provide excellent resolution. In addition, continued advances in NGS technology are beginning to provide a growing number of investigators access to the long reads necessary to generate high quality reference genomes. Data begets data and give rise to new applications of those data. The growth of public sequence databases and specialized pathogen tracking databases with detailed collection metadata inspire myriad new research activities into pathogen behavior, ecology, and evolution.

Over the past decade NGS enabled WGS has rapidly progressed from an experimental technology to a core technology for disease surveillance, response, and prevention. Exciting new applications of NGS technology beyond WGS are now being explored for their potential to augment outbreak response. Sophisticated metagenomic approaches are being increasingly explored as a potential means of

direct detection of pathogens in suspected contaminated substrate with the potential to speedup or bypass the culturing process. RNA-sequencing and ribosomal profiling allow for genome-wide investigation of transcriptional and translational activity which may eventually enable large-scale quantitative analysis of virulence factor expression across outbreak isolates. WGS has invigorated new research on pathogenicity and the future will hold new applications that will arise from this dynamic new technology. Sequencing technology continues to improve at a rapid rate. As the field continues to mature one can also expect to see sequencers emerge that can work with smaller inputs and provide actionable data with quicker turn around and reduced costs.

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Overview on the Systematics of Biotoxins as Threat Agents

15

Harald Striegl

15.1 Introduction

Biotoxins are neither distinct biological nor chemical agents in a common understanding but can be considered as 'mid-spectrum agents' [1–3]. As a matter of fact, they deserve special attention as a group of threat agents of biological origin with great potential to harm people [4]. There is a broad spectrum of biotoxins that can be used in biowarfare and in bioterrorist attacks. The spectrum of biotoxins ranges from peptides and proteins to alkaloids and other bioactive small molecules [5, 6].

On the one hand, biotoxins differ from chemical threat agents (CTA) since they are almost never produced synthetically, volatile gases or able to be absorbed through the skin. On the other hand, biotoxins differ from classical biological threat agents (BTA) because they do not carry any genetic information like bacteria or viruses. Nevertheless, some biotoxins are extremely toxic threat agents that can be dispersed as aerosols, liquids or as powders and consequently have the potential to create casualties, alteration or breakdown of social life, or economic loss if used in warfare or a terrorist attack [2, 7–9].

The focus of this chapter will be on biotoxins with mass casualty potential. The differences between CTA, biotoxins, and BTW are explained, and strong emphasis will be placed on the classification of these special group of agents. Biotoxins can be grouped into different 'classes' by mechanism of action or organism of origin [2, 10]. Below, the focus will be strictly on the classification according to the organisms of origin since these agents are very heterogeneous molecules. Additionally, the chapter provides a complete overview of biotoxins that have been considered as threat agents at a certain point by different credible international conventions.

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15.2 Biotoxins as Mid-Spectrum Agents

Paracelsus (1493–1541) expressed the toxicology maxim that "*all things are poison and nothing is without poison, only the dose permits something not to be poisonous*". His principle is based on the simple assumption that all substances can be toxic and "*the dose makes the poison*". The famous Paracelsus phrase also applies to biotoxins. Dose is the key parameter in the hazard identification and risk assessment of biotoxins and the harmful effect is associated with their toxic properties.

As chemicals of biological origin, biotoxins possess characteristics of both groups: chemical and biological agents [4]. Biotoxins are always produced by living organisms and have adverse health effects on humans or other organisms [3, 4]. They represent a subset of poisonous substances in general and can lead to a wide variety of pathologies. The diversity of biotoxins is enormous and includes an extremely heterogeneous group of substances from low-molecular-weight compounds to complex macromolecules [11, 12].

There are a number of reasons why some biotoxins should be considered as threat agents. Biotoxins are naturally occurring substances and their biological effects can cause serious injury or even death. That, in combination with the often existing lack of antidotes for post-exposure prophylaxis and treatment, vaccines for pre-exposure prophylaxis or detection methods makes these molecules critical.

Unlike bacteria or viruses, biotoxins are not able to reproduce themselves or to reproduce with the help of host organisms. Biotoxins do not carry the genetic information necessary for their own amplification and, in view of this fact, these substances resemble chemical agents. CTA, however, possess different characteristics than biotoxins and belong to various classes of compounds with distinct physicochemical, physiological, and chemical properties [13, 14]. Due to the diversity of molecular size and composition of biotoxins and the resulting different physicochemical, physiological and chemical properties they are mostly grouped according to the organisms of origin [2, 10].

Moreover, in contrast to classical CTA, almost all biotoxins are substances that have a low vapor pressure at room temperature. Many CTA—but not all—have a high vapor pressure, resulting in a low boiling point, which causes evaporation from a liquid or solid form to the surrounding air [13]. Since biotoxins are almost never volatile, they cannot be dispersed as gas in contrast to many classical CTA. From this physicochemical perspective, biotoxins are more closely related to classical BTA such as viruses and bacteria.

Beside this fact, the production processes of biotoxins are still completely different compared to those of CTA. Biotoxins are almost exclusively produced by living organisms, whereas CTA are per se synthetically manufactured [14, 15].

Another very distinct feature of biotoxins is that they cannot penetrate the intact human skin without the help of other substances. Dimethyl sulfoxide or other molecules can increase the ability of some biotoxins to penetrate through the skin, but most of them are not skin permeable per se. In contrast to that, some CTA—mustard gas for example—are very lipophilic agents, which can penetrate textiles, biological protective clothing, and even the intact skin.

A further very characteristic feature of many BTA agents, including biotoxins, is an active response of the immune system after contact with those substances. Due to their biological origin, biotoxins stimulate immune reactions. A large group of biotoxins are peptides or proteinogenic molecules that can interfere with the human immune system. The adaptive immune system reacts to most foreign biological substances in a specific way, and the next time the same molecule is encountered, the adaptive immune system can respond faster.

Production, volatility, skin permeability, and immunoreactivity enable the approach of a distinction between biotoxins and CTA. There are also several other indicators and selection criteria available to determine the chemical or biological affiliation of biotoxins (e.g., odor, taste).

The number of biotoxins that can be used as mass casualty biological weapon is very limited. On the one hand, some of the highly toxic biotoxins are not very stable and on the other hand, some of less toxic biotoxins cannot be produced in high quantity or delivered to cover large areas or surfaces [2]. Table 15.1 lists the main criteria, which allow a rough assignment of CTA, biotoxins, and BTA agents.

How difficult it is to distinguish biotoxins from CTA and BTA agents is shown by the following examples. Depending to the authorities involved, the protein ricin is considered as CTA or BTA or both. The organism of origin is the castor oil plant (*Ricinus communis*). Neither the molecular weight, the ability to trigger a clear immune response, nor the natural origin indicates ricin to be a CTA. However, the lack of genetic information for reproduction moves ricin into the direction of CTA.

Likewise, some CTA have characteristics of biotoxins or even BTA. Other CTA, however, are considered unambiguously chemical. An example is sarin, one of the most prominent chemical agents. Sarin is an odorless liquid, which can barely penetrate the human skin. This criterion seems to direct sarin to the BTA or biotoxin side. But as a low-molecular-weight molecule of synthetic origin, which can be produced in large quantities, it clearly fulfills the most important criteria of chemical agents. Therefore, sarin is a CTA and differs from biotoxins and classical BTA.

In summary, several criteria exist to distinguish between BTA, CTA, and biotoxins. However, these individual criteria are not a comprehensive list for the description of threat agents. In general they allow a rough classification of biotoxins in a separate agent group. But, not all criteria must necessarily be fulfilled to place a biotoxin into a particular group. Neither is just one single criterion a prerequisite, nor must several criteria automatically lead to a biotoxins grouping. Nevertheless, in general the criteria allow a classification and an objective comparison of most of the CTA, biotoxins, and BTA agents.

15.3 Committees and Bodies Dealing with Biotoxins

Biotoxins vary according to their organism of origin, molecular structure, size and mode of action. As indicated, not all biotoxins can be considered as mass casualty weapons because not all biotoxins can cause death or disease on a large scale. For

Table 15.1 Different criteria for the discrimination of biotoxins from CTA and classical BTA agents like bacteria and viruses

Criterion	CTA	Biotoxin	BTA
Carrier of genetic information	Never	Never	Always
Type of dissemination	Physical state varies (solid, liquid, gas)	Solid or liquid	Solid or liquid
Effect	Immediately	Mostly short latency period	Mostly long infection period
Immune response	Rare	Mostly immune response	Clear immune response
Infectivity	Not infectious	Not infectious	Often infectious
Molecular size	Low-molecular compounds	Heterogeneous substances (low molecular weight compounds to complex macromolecules)	Highly complex molecular structure
Odor	Characteristic odor	Usually odorless	Usually odorless
Origin	Synthetic	Natural	Natural
Production procedures	Mostly less complex	Mostly complex	Complex
Removal	Decontamination	Decontamination	Disinfection
Routes of entry into the body	Varies; All routes are possible	Via aerosol or oral	Via aerosol or oral
Skin/dermal penetration	Often	Very seldom	Usually none
Taste	Often characteristic taste	Mostly tasteless	Tasteless
Toxicity	High	High	Not toxic
Volatility	Often	None	None

Adopted from Franz [2], Madsen [4], Anderson [7]

this reason, different committees discussed the potential of some biotoxins to be used for biowarfare or bioterrorism.

The Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction, commonly known as the Biological Weapons Convention or Biological and Toxin Weapons Convention (BTWC), discussed biotoxins that do not have prophylactic, protective or other peaceful purposes or that can be used for hostile purposes or in armed conflict [16]. The BTWC was the first multilateral disarmament treaty banning a category of biotoxins [16, 17].

Although biotoxins are considered to be biological, they are still toxic chemicals. Hence, biotoxins are also addressed by the Chemical Weapons Convention (CWC). The CWC aims to eliminate an entire category of weapons of mass destruction by prohibiting the development, production, acquisition, stockpiling, retention, transfer or use of chemical weapons—including toxins weapons—by States Parties [18]. The

agents, which are explicitly specified in the convention for monitoring purposes, cover a wide range of compounds and include chemical warfare agents and biotoxins, including key and more distant precursors. These compounds, or families of compounds, are listed in the three schedules of the convention's Annex [19]. Schedule 1 comprises those agents that have been or can easily be used as chemical weapons and which are of limited, if any, uses for peaceful purposes. This list includes two biotoxins: ricin and saxitoxin [19].

Along with the international conventions on biological and chemical weapons, the US Centers for Disease Control and Prevention (CDC) have prepared a strategic plan for bioterrorism preparedness and response. The plan includes a list of selected agents with putative impact for the public health system. These critical CDC Bioterrorism Agents/Diseases were classified into three Categories: A, B or C. Categorization was based on different criteria like transmission capabilities, severity of morbidity and mortality, and likelihood of use [20]. Many of these agents, in particular biotoxins, are capable to contaminate food or water supplies.

Biotoxins can be found in CDC Categories A and B. Category A agents are the highest priority agents and include *Clostridium botulinum* toxin. This biotoxin is considered to pose a risk to national security as it can easily be disseminated and cause high lethality, with potential for major public health impact. An attack with this toxin might also cause public panic and social disruption and hence requires special action for public health preparedness [20]. Those biotoxins are supposed to be moderately easy to disseminate and cause moderate morbidity and low lethality. Category B agents are the second highest priority agents and include the plant toxin ricin. This biotoxin is considered moderately easy to disseminate; results in moderate morbidity rates and low mortality rates and requires specific enhancements of CDC's diagnostic capacity and enhanced disease surveillance [20].

Another plurilateral like-minded committee addressing questions on BTA and CTA including biotoxins is the Australia Group (AG). All of the participants of the AG are states parties to the BTWC [21]. The AG is an informal forum which, through the harmonization of export controls, seeks to ensure that exports do not contribute to the development of chemical or biological weapons. Coordination of national export control measures assists AG participants to fulfil their obligations under the CWC and the BTWC to the fullest extent possible [21]. One of the group's goals is to agree on agents which are critical for chemical and biological weapons proliferation programs.

Several additional national war weapons lists exist but there is no room to present all of them here (e.g., German *Kriegswaffenliste*, EU CBRN Action Plan). However, all of these conventions and lists (including the ones mentioned above) share a joint understanding and agree on the mass casualty potential of distinct biotoxins. To summarize, only around twenty biotoxins out of millions are considered as mass casualty biological weapons capable of causing death or disease on a large scale. Table 15.2 gives an overview of all of this high risk biotoxins.

Table 15.2 Biotoxins of high risk biological agents lists of the (not adopted) control protocol for the BTWC, the CWC, the AG, and the CDC

Biotoxins	Organism of origin	Class	Listed
Abrin	Rosary pea (*Abrus precatorius*)	Plant toxin	AG, BTWC
Aflatoxin	*Aspergillus flavus* among others	Mycotoxin	AG
Anatoxin	*Cyanobacteria*	Phycotoxin	BTWC
Botulinum toxin	*Clostridium botulinum* among others	Bacterial toxin	AG, BTWC, CDC
Bungarotoxin	Kraits (*Bungarus* snakes)	Venom	BTWC
Cholera toxin	*Vibrio cholera*	Bacterial toxin	AG
Ciguatoxin	*Gambierdiscus toxicus*	Phycotoxin	BTWC
Clostridium perfringens toxins	*Clostridium perfringens*	Bacterial toxin	AG, BTWC
Conotoxin	Cone snails	Venom	AG
Diacetoxyscirpenol	Several fungi	Mycotoxin	AG
Trichothecene toxins	Several fungi	Mycotoxin	AG, BTWC
Microcystine (Cyanoginosin)	Cyanobacteria	Bacterial toxin	AG
Modeccin	Wild granadilla (*Adenia digitata*)	Plant toxin	AG
Ricin	Castor oil plant (*Ricinus communis*)	Plant toxin	AG, BTWC, CDC, CWC
Saxitoxin	*Alexandrium catenella* et al.	Phycotoxin	AG, BTWC, CWC,
Shigatoxin	*Shigella dysenteriae*, *E. coli* among others	Bacterial toxin	AG, BTWC
Staphylococcus aureus toxins	*Staphylococcus aureus* among others	Bacterial toxin	AG, BTWC
Tetanus toxin	*Clostridium tetani*	Bacterial toxin	AG
Tetrodotoxin	Several marine animals	Phycotoxin	AG
Viscumin	Mistletoe (*Viscum album*)	Plant toxin	AG
Volkensin	Kilyambiti plant (*Adenia volkensii*)	Plant toxin	AG

15.4 Classification of Biotoxins

15.4.1 Animal Venoms

Biotoxins and mixtures of them are present in all branches of biological life. A large number of those biomolecule cocktails are found in the animal kingdom and are known as venoms. Animal venoms are heterogeneous blends of toxic substances—mainly of protein and peptide origin—used to hunt for prey or defend against enemies [22]. As a matter of fact, the functional mechanisms of these biological

cocktails are multifaceted and individual compounds of venoms can reinforce each other. Venoms interfere with enzymes, receptors, or ion channels, with impact on the central and peripheral nervous system, the cardiovascular and the neuromuscular system, blood coagulation and homeostasis [23]. In contrast to the harmful effect of venoms, specific compounds of venoms have been increasingly used as pharmacological tools and as prototypes for drug development [24, 25].

The extraction, processing and enrichment of venoms from animals for dissemination and use as threat agent are very challenging. Nevertheless, many of these biotoxins are somewhat accessible and in public perception. Indeed, two zoonotic toxins are listed in the above mentioned international agreements banning biological or chemical weapons: bungarotoxins and conotoxins.

Bungarotoxins are a group of neurotoxic proteins found in the venom of snakes of distinct species, the kraits (*Bungarus* spp.) [26–28]. Four different bungarotoxins are known to interfere with neurological processes: Beta-bungarotoxin acts pre-synaptically, gamma-bungarotoxin antagonizes binding of acetylcholine post-synaptically at peripheral neuromuscular junctions and kappa-bungarotoxin blocks neuronal nicotinic receptors. The most prominent member of the bungarotoxin group is alpha-bungarotoxin. It can lead to headache, unconsciousness, paralysis, respiratory failure, and even death. Alpha-bungarotoxin is a neurotoxin, first described in 1963. It blocks nicotinic acetylcholine receptors and is widely used in medical applications [29–31].

Conotoxins are of special interest for modern pharmaceutical research and are listed for control by the AG. These neurotoxic peptides are derived from cone snail venom and differ between individual snail species. The active components of conotoxins are typically 12–30 amino acid residues in length and act on a wide variety of ligand-gated ion channels leading to various symptoms including paralysis, respiratory failure, and coma [3, 32].

15.4.2 Bacterial Toxins

The biggest group of biotoxins with putative threat potential is the bacterial toxin group. Bacterial toxins can be differentiated into two major classes on the basis of several criteria e.g. their chemical structure, thermostability, and method of release as a pathogen: exotoxins and endotoxins [2, 6, 33].

Endotoxins are structural components of bacteria and part of their cell envelopes. They are bound to the cell wall of gram-negative bacteria and relate specifically to the lipopolysaccharides or lipooligosaccharides located in the outer membrane. Endotoxins may be released from lysed bacteria as a result of effective host defense mechanisms.

Exotoxins are secreted by bacterial cells into the surrounding environment during exponential growth but may also be released during lysis of the cell. The secreted

toxins, soluble proteins or polypeptides, are produced by particular gram-positive or gram-negative bacteria that trigger the disease associated with their respective toxins. All bacterial toxins listed on international agreements banning biological or chemical weapons are protein exotoxins.

Among these very important bacterial toxin group is the so called AB_5 toxin subset [34]. All bacterial toxins of this group contain an enzymatically active A subunit and a homopentameric B subunit which mediates cell entry by oligosaccharide recognition [34–36]. The most prominent AB_5 toxins are **shigatoxins** produced by *Shigella dysenteriae* type 1 and **cholera toxin** produced by *Vibrio cholerae*. Furthermore, **verotoxins** also belong to the group of AB_5 toxins since they are homologous to shigatoxins but produced by enterohaemorrhagic *Escherichia coli* [34, 37–39]. Interestingly, shiga- and verotoxins are structurally closely related to very important biotoxins from plants (e.g., ricin) and are also members of the same ribosome-inactivating protein family (see Sect. 15.4.5).

Further prominent representatives of the exotoxins are the botulinum and tetanus neurotoxins.

Botulinum Neurotoxins (BoNT) are extremely poisonous metabolic products of *Clostridium botulinum* and some other clostridiae and are considered as the most potent natural toxins known [40–48]. *C. botulinum* is a gram-positive, spore-forming rod-shaped bacterium. It grows under the exclusion of oxygen and releases neurotoxins into the surrounding medium.

Six phylogenetic distinct clostridiae are known to produce seven serotypically distinct BoNTs (A-G) [49]. Serotype H was previously discovered but also described as BoNT/FA or BoNT/HA since this serotype seems to be a hybrid of BoNT A und F [50–56]. Types A, B, E, and the rare types F and H are human-pathogenic [57–59].

C. botulinum is widely distributed throughout nature and can occur ubiquitously in soil and mud. Gastrointestinal and cutaneous transmission is possible, respiratory cannot be excluded [60, 61, 62–67]. The main source of human intake of botulinum neurotoxin is contaminated food, mostly meat and sausage products [60]. Depending on the amount of toxin absorbed, symptoms can already appear after a few hours. The toxic effect is caused by irreversible binding to presynaptic nerve endings stopping the release of acetylcholine, thereby disrupting neurotransmission. As a result, neuromuscular transmission is blocked leading to flaccid paralysis.

Tetanus Neurotoxin or tetanospasmin is a poisonous metabolic product of another clostridium: *Clostridium tetani* [68]. The gram-positive spore-forming cells produce the extremely potent neurotoxin under anaerobic conditions. Like *C. botulinum*, *C. tetani* is found throughout nature and can occur ubiquitously in nature. Nowadays, tetanus is a rare disease in the western hemisphere due to excellent vaccination coverage, nevertheless it is still widely distributed in other parts of the world and a major cause of neonatal death in non-vaccinated mothers [69]. The molecular mechanism of action of tetanus toxin results in spastic paralysis [70].

***Clostridium perfringens* Toxins** are other biotoxins with mass casualty potential produced by *C. perfringens*, an ubiquitous bacterium present in the gastrointestinal tract of humans and animals. The gram-positive, anaerobic, endospore forming, and rod-shaped bacteria produce a variety of toxins under anaerobic conditions [71]. These are classified into five 'toxinotypes' (A–E). Each of these toxinotypes is associated with many, often life-threatening illnesses. Especially *C. perfringens* epsilon-toxin, one of the most potent toxins known, is considered as a potential biological weapon and produced by toxinotypes B and D strains [72]. Epsilon-toxin belongs to the heptameric β-pore-forming toxins, which are characterized by the formation of a pore through the plasma membrane of cells, leading to perivascular edema and necrotic lesions causing neurologic signs [73].

***Staphylococcus aureus* Toxins** are biotoxins with mass casualty potential produced by *Staphylococcus aureus* [11]. The gram-positive, round-shaped bacterium can be found everywhere in healthy persons' normal bacterial flora; mostly on the skin, respiratory tract, mucous membranes and in the nose. Nevertheless *S. aureus* can also be very virulent and cause a variety of severe diseases [74, 75]. Some strains are able to produce highly heat-stable protein enterotoxins responsible for symptoms of food poisoning after intake of contaminated food [3]. Staphylococcal food poisoning leads to vomiting, nausea, stomach cramps, and diarrhea within a very short period of time (minutes to hours). The most important staphylococcal enterotoxin which may be used to construct a bioweapon is staphylococcal enterotoxin B (SEB) [3, 4, 76].

15.4.3 Marine Toxins

Marine toxins, also known as phycotoxins, are a very heterogeneous group of biotoxins. They include, for instance, alkaloids, amino acids, and polyketides. They are a class of highly diverse compounds in terms of both structure and biological activity [77]. Phycotoxins can cause various clinically described syndromes, characterized by a wide range of amnesic, diarrheic or azaspiracid symptoms [78]. They cause paralytic shellfish poisonings and ciguatera fish poisoning [78, 79]. Some of these toxins are putative threat agents and almost all members out of this group interfere with neurological processes. They interact with ion channels or receptors, leading to different neurotoxic symptoms and even death. Generally, these types of neurotoxins are marine toxins produced primarily by phytoplankton e.g. flagellates and diatoms, but also by several types of cyanobacteria, invertebrates or other organisms [77].

Most of the phycotoxins that have been considered as threat agents are produced by cyanobacteria (microcystin, anatoxin and saxitoxin). Cyanobacteria—a phylum of bacteria—are ubiquitous photosynthetic microorganisms forming blooms and scums in surface water. Among them, several are known to produce cyanotoxins

giving rise to concern for human health. Cyanobacteria are prokaryotes obtaining energy via photosynthesis. This selling proposition makes cyanobacteria very unique and allows us to separate cyanotoxins from other bacterial toxins.

Microcystines are cyclic peptides produced by a group of cyanobacteria, mostly *Microcystis* spp. Several different microcystins exist and all consisting of a seven-membered peptide ring, which is made up of five non-natural amino acids and two natural amino acids [3]. These natural amino acids distinguishes microcystins from one another, while the other amino acids are more or less constant [3]. Microcystins can cause acute poisonings with a variety of different symptoms and sometimes fatal outcome, but also cancer [80, 81].

Anatoxins are other marine phycotoxins produced by cyanobacteria in the *Anabaena* genus worldwide [82–84]. The most important is anatoxin-a, also known as *Very Fast Death Factor*, which is a secondary amine. Other structurally related alkaloids are homoanatoxin-a, as well as anatoxin-(a)s a unique *N*-hydroxyguanidine methyl phosphate [85–88]. Intoxication by anatoxins results very rapidly in neurotoxic effects, which is specific for this group of phycotoxins.

Saxitoxins are also marine phycotoxins produced by cyanobacteria and dinoflagellates, listed by schedule 1 of the CWC. The saxitoxin-group corresponds to toxic metabolites produced by cyanobacteria and dinoflagellates of the genera *Alexandrium*, *Gymnodinium*, and *Pyrodinium* [89]. Oral uptake of the quite stable saxitoxin and its derivatives can lead very rapidly to paralytic shellfish poisoning including gastrointestinal and neurological signs symptoms [90–92].

Ciguatoxins are a different marine phycotoxin group causing fish poisoning. These toxic polycyclic polyethers are manly produced by the dinoflagellate *Gambierdiscus toxicus* in the Pacific. The dinoflagellates accumulates in fish through the food chain and causes the complex ciguatera clinical picture, including paralysis, heart contraction, and changing the senses of heat and cold. The mechanism of action is the interference of ciguatoxin with voltage-gated sodium channels in synapses of the nervous system [78, 91, 93–95].

Tetrodotoxin is another marine phycotoxin that is considered a potential threat agent [96]. The neurotoxin has been isolated from animals of widely differing species [97]. Tetrodotoxin is well known because of its accumulation in the pufferfish (Fugu), which is a Japan delicacy. The fish must be processed extremely carefully to remove toxic parts containing tetrodotoxin to avoid poisoning. The toxin inhibits the firing of action potentials in neurons by binding to the voltage-gated sodium channels in nerve cell membranes and blocking the passage of sodium ions into the neuron [96]. Symptoms develop very rapidly (within minutes) and include facial and extremity paresthesias and numbness, which may be followed by dizziness and profuse sweating. Death can takes place within a few hours.

15.4.4 Mycotoxins

Mycotoxins are a large group of diverse secondary metabolites produced by a wide variety of filamentous fungi [98]. Up to 400 different molecules are known to be part of the mycotoxin group [99]. Molds of several species may produce the same mycotoxin but sometimes one mold may produce many different mycotoxins [100]. All mycotoxins are low-molecular-weight molecules with the potential to induce toxicological effects in humans and other vertebrates and many mycotoxins display overlapping toxicities to invertebrates, plants, and microorganisms [101]. Mycotoxins are mostly known to cause food poisoning [102].

Trichothecene mycotoxins are produced by several fungi, especially those of the *Fusarium* genus [7, 98]. They have been classified into four groups (Types A, B, C, and D) based on the structure of the molecules [103–105]. Type A-trichothecenes are of special interest in regard to toxicity. They include toxins such as mono- and **diacetoxyscirpenol**, HT-2 toxin, T-2 toxin or neosolaniol [103]. However, some members out of the type B-group also have the potential to harm people in a bioterrorist attack (e.g., deoxynivalenol known as vomitoxin). Trichothecenepoisoning can lead to a variety of clinical signs, including weakness, ataxia, hypotension, coagulopathy, and death [106].

Aflatoxin mycotoxins are a group of chemically similar metabolites produced by certain fungi of the genus *Aspergillus* [98]. Aflatoxins are polycyclic aromatic compounds (difuranocoumarins). Several types are produced in nature and four aflatoxins (B_1, B_2, G_1, and G_2) are naturally found in foods. The predominant site of aflatoxin metabolism is the liver (cytochrome p450 enzymes). There, the biotoxins are metabolized into highly reactive exo-epoxides. Aflatoxin B_1 is most commonly found in food and the most toxic out of the aflatoxin group. Aflatoxins can cause acute poisonings but they are also very potent carcinogens and mutagens casing chronic clinical signs and hepatocellular cancer [107, 108].

15.4.5 Plant Toxins

Extremely toxic biomolecules are biotoxins produced by different plants. Countless plant toxin effects are known since ancient times. Even the father of Greek philosophy, Socrates, died from a plant toxin when he drank a cup of poisonous hemlock. Remarkably, just only a single plant toxin group out of several different has been considered as weapons at a certain point by different committees: the ribosome-inactivating proteins (RIPs) [109].

RIPs are known to be produced by several organisms of all kingdoms: bacteria, fungi, algae, plants, and animals (see Sect. 15.4.2: shiga- and verotoxins). This group of proteins irreversibly modifies ribosomes via their adenine polynucleotide glycosylase activity on different nucleic acid substrates. These modifications are responsible for the arrest of protein synthesis leading to cell death. RIPs have been

classified as type 1, 2, and 3. Type 1 RIPs are single-domain proteins that contain an *N*-glycosidase activity. Type 2 RIPs form a heterodimeric complex consisting of an A-chain and a B-chain linked by disulfide bounds [110, 111]. The A-chain is functionally equivalent to type 1 RIPs (A-chain) but is fused to a C-terminal lectin domain (B-chain). Lectins are glycoside-binding proteins which via lectin-carbohydrate interactions allow the holotoxin to bind to the cell surface. Type 3 RIPs are very rare, only a few of this structurally different RIP types have been classified so far [110, 112, 113].

In general, type 2 RIPs are several times more toxic than type 1 and 3 RIPs, although exceptions are possible (e.g., nontoxic type 2 RIPs) [113, 114]. Only type 2 RIPs, namely abrin, modeccin, ricin, viscumin, and volkensin are agents of concern recognized by committees. Modeccin, viscumin, and volkensin are listed by the Australia Group for export control, but abrin and ricin are considered as dangerous by bodies [115]. Depending on the manner of intoxication, toxicity varies and clinical signs differ.

Ricin is a type 2 RIP produced primarily in the seeds (castor beans) of the castor oil plant (*Ricinus communis*), a member of the spurge family Euphorbiaceae [115]. The plant is native to Africa and cultivated all over the tropical and subtropical world. It is often grown as an ornamental annual in temperate zones and commercially cultivated because of its high amount of oil (castor oil) within the beans which is mainly used in clinical and industrial processes. At the cellular level, ricin hydrolyses the *N*-glycosidic bond of the adenine residue A4324 within the 28S rRNA and leaves the phosphodiester backbone of the RNA intact [116, 117]. Depending on the manner of intoxication, toxicity varies and clinical signs differ. Oral intoxication mostly leads to severe gastrointestinal signs, whereas intoxication by inhalation can cause circulatory instability and severe lung damage.

Abrin is a highly toxic type 2 RIP [115] several times more toxic than ricin. The protein is found in the seeds of the rosary pea (or jequirity pea from *Abrus precatorius*). At the cellular level abrin, causes protein synthesis inhibition at the same site as ricin [118]. Identical RNA *N*-glycosidase activity is present in **modeccin**. This plant type 2 RIP is produced by wild granadilla (*Adenia digitata*) [119]. The fruit and roots are known to be used for suicide. *Adenia* is a genus of flowering plants in the passionflower family, Passifloraceae. The kilyambiti plant (*Adenia volkensii*) is another member of this genus and family that produces a type 2 RIP, **volkensin**, in its roots [120]. Finally, **viscumin** is a toxic type 2 RIP from mistletoe (*Viscum album*) [121].

15.5 Conclusion

Special attention must be paid to ‘mid-spectrum agents’ that pose a serious risk as threat agents or weapons. Besides biotoxins, several other mid-spectrum agents are known. Bioregulators for example are—like biotoxins—on the borderline between

'synthetic' and 'natural' and are neither clear distinct chemical nor biological agents. They are also naturally occurring agents lacking genetic information and are produced by living organisms in order to regulate diverse cellular processes. Like biotoxins bioregulators can have adverse health effects on humans in a short period of time if they are used as biowarfare and bioterrorism agents.

'Mid-spectrum agents' of biological origin have been considered as weapons or instruments of terror. It is impossible to enumerate all molecules of biological origin that have influenced warfare or terroristic efforts or even may be used for such purposes. However it remains to be emphasized that in the case of biotoxins; only around 20 have been discussed in the public by different credible international conventions or bodies as founding substances for weapon capable of causing death or disease on a large scale. Thus, at least these biotoxins ought to be discussed further in regard to challenges and requirements with respect to public health preparedness. The biotoxins discussed in this chapter may serve as the basis for the development of appropriate methods of management and countermeasures, including decontamination and Personal Protective Equipment strategies.

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Agrobioterrorism

16

Lawrence F. Roberge

16.1 Introduction

The importance of food and agriculture to mankind can be traced back over 10,000 years ago. Yet, in this modern age of biological weapons, agriculture and agricultural products have been targeted by various nation states as viable strategic targets and targeted by terrorists (aka non-state actors) for acts of bioterrorism [1, 2]. This paper will examine the reasons for biological weapons (BW) to be targeted at food and agricultural systems and the history of the development of agricultural BW. The term "agrobioterrorism" (also referred to as "agricultural bioterrorism"), can be defined as the use of pathogens or toxins against agricultural products or facilities usually with the resultant effects of causing casualties or fatalities from contaminated agricultural resources or foodstuffs. Chalk defines agroterrorism "as the deliberate introduction of a disease agent, either against livestock or into the food chain, for purposes of undermining socioeconomic stability and/or generating fear." Chalk also notes that agroterrorism can be used "either to cause mass socioeconomic disruption or as a form of direct human aggression" [3].

This paper will discuss the economic and national security concerns over the use of BW on food supplies or agricultural production. This paper will also examine some of the technologies and strategies regarding the development, detection, and containment of terrorist or national BW attacks against food or agricultural resources and counterstrategies.

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16.2 Reasons for Biological Weapons Attack

Horn and Breeze [4] briefly described how agriculture is one of the pre-eminent foundations for the United States' (US) wealth in the global marketplace and a key element for national security as part of US critical infrastructure. The US food and fiber system accounts for 13% of gross domestic product (GDP) and for 16.9% of total employment [4]. Agricultural exports alone account for $140 billion and for 860,000 jobs. The United States has been known to have one of the most safe, secure, and reliable supply of food at a reasonable price that the world has ever known. Finally, the authors note that only about 2% of the population is involved in agriculture with the remaining population available to engage in business, commerce, and other wealth creating endeavors [4].

Yet, as Brown points out [5], much of the success in agricultural productivity and trade is dependent on freedom from disease. If a pathogen enters the food production arena, both the consumer and the export markets are adversely affected. The distribution of the pathogen would affect the consumer with increasing food prices (especially as contaminated food stocks were recalled from shelves or culled from infected farms), while a simultaneous drop in export-market transactions would occur as nations refuse to import food stocks to prevent the spread of the pathogen to their own farms or morbidity or mortality of their own populace. Two brief examples warrant mention here.

Brown notes that the last major foreign animal disease outbreak in the US was avian influenza (1983–1984) in Pennsylvania and several neighboring states. After the expensive eradication of infected chickens and decontamination of chicken facilities was completed, the cost of the process was $63 million which was paid out by the US federal government. During the 6 months period of the outbreak, the US consumer suffered poultry price increases to the total of $349 million [5]. The impact on Great Britain due to bovine spongiform encephalopathy (BSE) was even more stunning. The emerging disease in cattle (prion-based) required a mandated destruction of approximately 1.35 million cattle with all carcasses disposed of by incineration. This resulted in an estimated cost of over US$4.2 billion. Yet, as Brown notes, the cost in allowing prions into the food supply would have been devastatingly negative to the beef and dairy industries as a whole [5].

Parker [6] describes the "economic multiplier effect" of farm commodities as a measure of total economic activity of that commodity (e.g. eggs, grain, meat, milk). This multiplier effect starts at the farm gate value of the commodity and accrues value from transportation, marketing, and processing of the commodity. Parker states that the US Department of Commerce has concluded that the economic multiplier effect of exported farm commodities is 20 to 1 as compared to less than 2 to 1 for domestic crop sales and less than 3 to 1 for domestic livestock sales [6]. It is this multiplier effect which helps to account for US agricultural product exports constituting 15% of all global agricultural exports and making the farm component of the economy the largest positive contributor to the US trade balance [6].

The reasons for a BW attack on agriculture can be summarized by Chalk [3] who writes that three major outcomes would result from a bioterrorism attack on

agriculture. First, economic disruption would occur creating at least three levels of costs. Initially these costs come from eradication and containment measures. For example, during the 1997 outbreak of foot and mouth Disease (FMD) in Taiwan, the vaccination costs were $10 million, but the surveillance, cleaning, disinfection and related viral eradication costs were $4 billion. Second, costs are the indirect multiplier effects that would accumulate from both compensations paid to farmers for destruction of agricultural commodities and the revenue losses by direct and indirectly related industries (e.g. dairy processors, bakeries, abattoirs). Third, international trade costs would occur due to protective embargoes imposed by major export partners. One example is the 1989 Chilean grape scare caused by anti-Pinochet extremists that laced fruit bound for the US with sodium cyanide. While only a small handful of grapes were contaminated, the resultant imports suspensions (imposed by such nations as Canada, United States, Denmark, Germany, and Hong Kong) cost Chile over US$200 million in lost earnings [3].

Another possible outcome from a BW attack on agriculture would be the loss of political support and confidence in the government. Chalk [3] details how sociopolitical events, if not carefully controlled (including the media), would undermine the public's trust and cooperation in state and federal governance during the crisis. It is possible that euthanizing large numbers of animals to control the outbreak would result in such public distain that public protests could result to save infected animals or generate active resistance by farmers striving to protect infected herds from eradication [3]. These public reactions could leave politicians with little strength to follow the necessary protocols to contain the epidemic lest they are voted out by an angry albeit poorly educated populace. Chalk provides an example of the 2001 FMD outbreak in Great Britain that triggered a massive public resistance to the livestock eradication and thereby resulted in a tremendous loss of public support for the Blair government and the Labor party in general.

The next outcome of a BW attack on agriculture is based on the motive of all terrorist attacks; to elicit fear and anxiety among the public. Chalk [3] mentions the effects could include socially disruptive migrations from rural to urban to escape the possibility of a zoonotic pathogen "jumping" species and leading to a human epidemic. This could be further complicated if the pathogen did in fact jump the species barrier, or if it was genetically engineered to jump the barrier and infect humans and livestock. Chalk describes the example of the 1999 Nipah virus disease outbreak in Malaysia which not only destroyed the swine population of Negri Sembilan State, but also killed 117 villagers. During the height of the outbreak, thousands of people deserted their homes and abandoned livestock while becoming refugees in shanty towns outside of Kuala Lumpur [3]. It must also be mentioned that a highly organized terrorist group could use social anarchists to help incite further social chaos by following the food attacks with riots over food shortages or price spikes. The scenario could be seen as step one: attack food stocks; step two: incite fear and terror in the populace; step three: orchestrate protests and riots against the government that the public does not trust; step four: cause violence during the riots to galvanize further mistrust of the government and cultivate further social chaos.

Chalk finally discussed another outcome of a BW attack on agriculture: raising financial capital or blackmail. One possible route for a BW terrorist to raise financial capital would be to direct attacks which create and exploit fluctuations in the commodity futures markets. These attacks could be directed at crops or livestock—or even with the rise of biofuels—be directed against crops used for biofuels (e.g. corn or sorghum for ethanol production and soybeans or palm oils for biodiesel production). Either under direct support by other parties (e.g. organized crime, terrorists, foreign cartels) or acting independently, the BW terrorist would be able to take advantage of market reactions to the attack (as Chalk eloquently stated "allowing the 'natural' economic laws of supply and demand to take effect") and harvest maximum dividends from the commodity futures sales [3].

Chalk [3] also observed that this form of BW terrorism could make it easier for state and federal government officials to negotiate with the terrorists (extortion and blackmail) to avoid the immediate and latent effects of the attacks. These forms of attacks would not garner the same public outcry over dead farm animals as they would have had over *Bacillus anthracis* or variola virus attack with numerous human causalities.

Finally, Hickson [7] discussed the use of BW against "soft targets" as a form of Fabian strategy of indirect warfare. In essence, Hickson described the Fabian strategy (named after the Roman general Quintus Fabius Maximus, who defeated Hannibal by avoiding direct conflict) as a strategy of indirect actions used to weaken the resistance of an opposing force. For example, if an aggressor wishes to defeat an enemy; but to avoid the "after effects" of prolonged direct warfare that would leave deep scars on the civilization or the subsequent peace; then the aggressor must develop ways to weaken the enemy beyond their capacity to fight. This strategy could include BW directed at agricultural targets with the resultant effects of reduced export trade of agricultural commodities, food shortages, reduced employment for workers in agricultural and food related industries, reduced biofuels productivity (if the targets include biofuels crops), and due to the multiplier effects, a decreased economic vigor of the entire nation. This would result in a subsequent cascade of socio-economic effects, including as discussed above, distrust and resistance to state or federal government authority; greater social dissent exemplified by public protests over food or fuel shortages and spiking food prices; or riots over unemployment and food shortages. These final actions could indicate to an aggressor that the enemy is now weakened sufficiently so that a quick invasion and defeat is possible.

16.3 History of Biological Weapons Development or Attacks Against Agricultural Targets

Whether it is a nation sponsored or non-state sponsored (e.g. terrorist) BW attack against agriculture, it is important to understand the historical development of this weapons strategy. Although this paper cannot cover all historical aspects of the topic, it is important to mention various nations that did research or made advances

in the use of BW against agriculture and mention the use by terrorists against livestock, crops, or food.

In World War I, early uses of BW on agricultural targets involved German spies using of *B. anthracis and Burkholderia mallei* against pack animals (horses and mules) being shipped out for use in war. Anton Dilger, a German-American physician, cultured *B. anthracis and B. mallei* and had German agents or sympathizers infect the animals in stockyards prior to export to Europe [2, 8, 9]. Dilger's agents either injected the pathogens into the horses or added the pathogens into animal feed and/or the water supplies [2, 8, 9].

During World War II, Nazi Germany began extensive work on BW for livestock using rinderpest virus and FMDV and an array of anti-crop pathogens and pests [4, 10, 11]. Although twice during the war, Hitler forbade offensive BW development, research continued with German development of anti-crop weapons such as Colorado potato beetles, turnip weevils, pine leaf wasps, wheat blight, wheat rust, turnip fungus, potato stalk rot, potato blight (*Phytophthora infestans*), and smothering weeds [10, 11]. Some research demonstrated a successful means to disseminate fungal spores mixed in combination with talcum powder [4, 10, 12]. It must be noted that upon the defeat of France by Nazi Germany in 1940, Germany obtained a great deal of BW information from debriefing French BW researchers [10, 11].

France anti-crop program was mostly directed at Germany [4]. In 1939, French researchers explored methods to breed potato beetles and undertook release trials of the insects. Also, the French researched rinderpest virus and *B. anthracis* against livestock and performed research on potato blight [4, 10].

Japan, well known for the brutal use of BW against civilians and prisoners in China, was also actively researching and developing anti-crop and livestock BW [4, 10, 13]. Harris discussed the Japanese camp, Unit 100 (aka the Hippo-epizootic Unit of the Kwantung Army), which focused on animal and crop BW research [13]. The camp contained several farms, some of which grew poisonous plants thought to kill humans and animals or both. Other research done at these farms included development of herbicides used to kill plants or poison food. Also, the Japanese researched a variety of fungi, bacteria, and nematodes on most grains and vegetables grown in the regions of Manchuria and Siberia [4, 10]. Japanese researchers had limited success in the aerial dissemination of anthrax and glanders [13].

During World War II, the United States, Britain, and Canada were actively engaged in research and development of BW and eagerly exchanged technical information and research results [12]. The US gained much from Britain's research, especially from British researcher, Paul Fildes. Fildes and associates established the inhalation doses required to achieve infection in laboratory animals. Fildes and his colleagues also developed the means of using a high explosive chemical warfare munitions to create an aerosolized bacterial cloud of particles capable of remaining in the lung (e.g. anthrax). With research done at Porton Down, Britain developed a retaliatory BW capability that included the production of 5,000,000 anthrax-laced cattle cakes. These cakes were intended to undermine the agricultural sector of the German economy [12].

Biological weapons research in the United States during World War II included the development of anti-crop chemicals which were defoliants: 2,4-dicholorophenoxy acetic acid (2,4-D) and 2,4,5-tricholorophenoxy acetic acid (2,4,5-T) [10, 12]. Further research in anti-crop agents were directed at the pathogens: *P. infestans* (potato blight), *Athelia rolfsii* (sclerotium rot of sugar beets), *Magnaporthe oryzae* (rice blast), and *Cochliobolus miyabeanus* (brown spot in rice). This research was also directed at the use of resistant fungi and the development of more virulent fungal strains to enhance success of an attack even during adverse conditions of warfare [12].

During the 1950s and 1960s, the US directed the anti-crop research, conducted at the Crop Division at Fort Detrick, to mass production and storage of anti-crop agents. The research also included the development of delivery vehicles including a "feather bomb" consisting of a modified propaganda bomb loaded with feathers dusted with fungal spores. Upon release, the bomb was found to create 100,000 foci of infection over a 50 square mile area. Other anti-crop dispersal devices included large volume spray tanks to disperse dry anti-crop BW agents which could with one aircraft disperse a plant disease epidemic over an area in excess of 1000 km^2. Another dispersal device was a balloon gondola unit which could under the proper weather conditions would carry five containers of feather/fungal spore payloads deep into enemy territory. During this time period, various studies targeted the "grain belt" of Russia and the rice production regions of Communist China [10, 12].

The Soviet Union was known to have one of the most innovative and broad anti-crop and anti-livestock programs [4]. According to Alibek [14], the anti-crop program only began in the late 1940s or early 1950s. The anti-crop agents developed included wheat rust, rice blast, tobacco mosaic virus, brown leaf rust, and rye blast [4, 15]. The anti-animal (anti-livestock) agents included African swine fever virus, rinderpest virus, FMDV, vesicular stomatitis Indiana virus, avian influenza A virus, and a combined class of anti-personnel/anti-animal agents that included *B. anthracis* and *Chlamydia psittaci* (psittacosis) [12]. Mostly the anti-crop agents were targeted at US and Western European crops.

The Soviets were successful in lyophilization and vacuum storage of maize rust and other stabilization techniques for Newcastle disease virus [4]. The Soviets had claimed to have perfected insect rearing techniques and had built an automated mass rearing facility which would produce millions of parasitic insects per day [4]. The release patterns and dissemination of insect attractants were studied to influence the migration patterns of natural and deliberately introduced insects [4]. It must further be noted that despite signing the Biological Toxins and Weapons Convention (BTWC) treaty in 1972 and publicly renouncing BW research and development, the Soviets cheated on the treaty and continued weapons development and research well into the early 1990s [16].

After the fall of the Soviet Union, concern has been raised over the end of research into BW agents, including anti-crop weapons [14, 16]. Also, there has been anxiety that former Soviet BW scientists may transfer their knowledge or skills to rouge nations or terrorists [12, 14, 16]. As such, concern grows that the BTWC

may need further updates in the area of plant pathogens to clarify and monitor "peaceful" applications as opposed to BW applications [10].

In Iraq, research was pursued prior to the Persian Gulf War and focused mostly on wheat stem rust, camel pox, and anthrax [4]. In 1988 near Mosul, large field tests demonstrated that wheat fields could be infected with a fungal plant pathogen (*Tilletia indica* that cause wheat bunt, aka Karnal bunt). It must be noted that common bunt replaces the wheat seeds with black teliospores and the teliospores produce a gas, trimethylamine, which can cause explosions in wheat harvesters [17]. According to the United Nations Special Commission on Iraq (UNSCOM), this contaminated wheat crop was harvested and stored to be used as an "economic" weapon against Iran to cause food shortages [12] during the Iraq-Iran war (1980–1988).

Kolavic et al [18] and Carus [19] reported that in late 1996, Diane Thompson, a medical laboratory technician, deliberately contaminated pastries with *Shigella dysenteriae* type 2 bacteria and arranged to have them consumed in a break room by fellow laboratory workers. This act resulted in 12 laboratory workers becoming ill and one family member of one lab worker, who consumed the shared pastry at their home, also became ill. Although there were no fatalities, four victims required hospitalization and five others required emergency room treatment. The strain of bacteria was obtained from the medical laboratory's stock culture freezer where Thompson had ready access. Thompson was eventually arrested and sentenced to 20 years for intentional food tampering.

In 1989, a group calling itself "The Breeders" announced that they had bred and released Mediterranean fruit flies (*Ceratitis capitata*) to protest the use of pesticides in the southern California area [4, 20]. Later a United States Department of Agriculture (USDA) study identified peculiar patterns of Mediterranean fruit fly (Medfly) infestations, especially in new and strange places where the fruit fly would not likely appear. A review panel which included USDA scientists, concluded that someone or group was in fact breeding and releasing Medfly larvae. Later follow up attempts to communicate with the group yielded no criminal leads and no one to date has come forth or been apprehended over the incident [20].

Finally, Neher described an experience in the state of Wisconsin in late 1996, where an unknown person or persons notified the local police chief that animal feed products leaving a rendering plant were contaminated with a pesticide and to expect large-scale animal deaths [21]. Neher, then an administrator in the Wisconsin Department of Agriculture, Trade, and Consumer Protection, discussed how a Toxic Response Team was mobilized, analyzed records and samples, and determined within 2 days that the feed and liquid fat were contaminated with chlordane (an organochlorine pesticide). Due to excellent feed industry and government agency cooperation, all potentially contaminated feed was removed from major customers and the contaminated feed was replaced within 2 days. The recall of the feed and liquid fat resulted in the disposal of 4000 tons of feed and 500,000 pounds of fat with an estimated value of about $4 million. Although the terrorist was never caught, no livestock animals were found to have serious contamination, and no human causalities occurred [21].

16.4 Routes of Food

The movement of food and agricultural products into the food chain of US consumers (or most other nations) starts at the farm. Yet, the simplistic view of farmer's produce to consumer table has become quite complex in the later part of the twentieth and now twenty-first Century. Schwab [22] described how the US food system has gone away from the local farm to massive cooperate farms and that fact that the US food system is tied into the global food supply. The route for plant-based foods may include farm to warehouse/distribution center to grocer-produce section and then to the consumer table. BUT, if any modifications of the food product occur (e.g. milling, dehydration, packaging), then the farm delivers the product to a food processing factory. For livestock-based foods, processing includes the farm, abattoir (slaughterhouse), packing facility for additional processing (usually a factory department within the slaughterhouse), warehouse distributor, grocer, then to the consumer. Parker noted that each level of processing is a level of vulnerability in food BW attacks [6]. Cameron and Pate described how the US cultivation of certain crops are concentrated in certain areas of the country (example: in 1997, 75.5% of strawberries, 92.2% of grapes, 47% of tomatoes 33.8% of oranges for the US were grown in California) and as such, these areas would be more vulnerable to BW attacks [23].

Chalk also mentioned that developments in the farm-to-table continuum have greatly increased the points of entry for BW agents (for example: bacterial, viral, or toxin-based agents) [3]. Many of these processing and packing plants lack security and surveillance and Chalk notes that these factors augment the ease for a food-borne attack [3].

Schwab noted that food-borne diseases could be introduced into the food chain as another form of agricultural BW [24]. Food-borne or waterborne pathogens could be introduced in the market place (e.g. grocery store or restaurant) or during food processing. Because many food-borne pathogens can be found locally in soils, water, plants, and animals; a deliberate BW attack using these organisms might be overlooked as a BW attack and merely attributed to food contamination of one type or another. The BW attack using food or water is a preferred method as dispersal of a pathogen in food or water allows for the increased likelihood of affecting a greater number of people [24]. Schwab noted that the Centers for Disease Control and Prevention (CDC's) classification of Bioagents that are food-borne or waterborne include: Category A-Botulinum toxin; Category B-*Salmonella* species, *Shigella dysenteriae*, *Escherichia coli* O157:H7, *Vibrio cholerae*, *Cryptosporidium*, and noroviruses [24]. Finally, it must be noted that food- and water-borne pathogens have selective characteristics that favor their use as a BW agent such as low inoculation dose, ease of secondary transmission, and moderate to high persistence in the environment [24].

In a study by Wein and Liu, that was controversial over its public release, the researchers used a mathematical model that covered cows to consumers and analyzed a hypothetical bioterrorist attack using botulinum toxin deliberately

Table 16.1 Conclusions from a hypothetical bioterrorist attack using botulinum toxin deliberately released into a milk supply chain with a single milk-processing facility [25]

1. Due to dilution factors along the milk supply chain, a minimum amount of the toxin would be required to ensure consumer causalities
2. If terrorists obtained the proper amount, the rapid distribution and consumption would result in several hundred thousand causalities (NOTE: casualties of children due to their greater consumption of milk and greater toxin sensitivity would be significant)
3. The higher the initial dose of toxin introduced could mean a shorter time span for detection of poisoned milk as casualties began to appear more rapidly in the population
4. Current processing methods for milk-pasteurization-using either radiation or heat treatment are inadequate to inactivate the botulinum toxin, although Ultra High-Temperature (UHT) pasteurization (which has not been embraced by US consumers) will inactivate botulinum toxin in milk
5. An ELISA test for the toxin is available and if implemented would cost less than one cent per gallon of milk
6. More security measures for transport trucks, tanks, and silos and security background checks for farm laborers, plant personnel, and truck drivers are warranted as the present Food and Drug Administration (FDA) security guidelines are purely voluntary

released into a milk supply chain with a single milk-processing facility [25] (Table 16.1).

The authors concluded the following effects from the milk BW attack. First, due to dilution factors along the milk supply chain, a minimum amount of the toxin would be required to ensure consumer causalities. Second, if terrorists obtained the proper amount, the rapid distribution and consumption would result in several hundred thousand causalities (NOTE: casualties of children would be higher due to their greater consumption of milk and greater sensitivity to the toxin). Third, the higher the initial dose of toxin introduced would result in a shorter time span for detection of poisoned milk as casualties began to appear more rapidly in the population. Fourth, current processing methods for milk-pasteurization using either radiation or heat treatment are inadequate to inactivate the botulinum toxin, although Ultrahigh-Temperature (UHT) pasteurization (which has not been embraced by US consumers) will inactivate botulinum toxin in milk. Fifth, an ELISA test for the toxin is available and if implemented would cost less than one cent per gallon of milk. Sixth, more security measures for transport trucks, tanks, and silos and security background checks for farm laborers, plant personnel, and truck drivers are warranted as the present Food and Drug Administration (FDA) security guidelines are purely voluntary [25].

16.5 Modes of Attack

Parker described five potential targets of agricultural bioterrorism: field crops, farm animals, food items in the processing or distribution chain, market-ready foods at the wholesale or retail level, agricultural facilities that include processing plants, storage facilities, and components of the transportation sector and research laboratories

[6]. Parker noted that most concerns over agricultural bioterrorism (or biological warfare attacks from nation states) have focused on mostly on field crops and farm animals. Yet, Parker noted that it is critical to be aware that BW attacks against foods in the food chain and also notes that research facilities engaged in investigations or analysis of foods could also be targets of attack [6]. Von Bredow et al. [26] noted that compared to human food, one of the most vulnerable (i.e. least guarded) sources of the food supply is animal feed. Von Bredow et al. noted that considering the vast amount of feed required by poultry and livestock, it would be next to impossible to secure all of this food [26]. Yet, it is possible that by contamination of the animal feed, the contamination could easily end up in the human food chain (as exemplified by the Wisconsin bioterrorism case described by Neher [21] above).

Kosal and Anderson [27] described an incident during which an antibiotic feed additive, salinomycin, a lipid soluble ionophore that is contraindicated for camelids (such as alpacas), was unknowingly added at the commercial feed production facility. The feed was distributed to 6–8 alpaca farms resulting in over 1000 alpacas exposed and 135 deaths. After confirmation of the salinomycin contamination by the Ohio Department of Agriculture, a recall of the contaminated feed was issued. This is an example of how a contaminant at a nexus point of manufacture can have far reaching effects in the agricultural food chain.

A brief review of various agents of attack, based in part on previous BW weapons researched or developed for agricultural targets and discussion of bioterrorism will be described below.

16.5.1 Crops

Most crop pathogens in BW research have been chosen due to their ease of culture, stability in storage, ease of dissemination, and capacity to cause significant damage over a rapid period of time [10]. From a phytopathologist's view, the variety of pathogens for any plant includes viruses, nematodes, bacteria, fungi, mycoplasma, and insects either acting as vectors or as crop pests. Furthermore, even with the successful dispersal of pathogens, other environmental conditions can affect the chances of the pathogen causing an epidemic in the crops. These variables include: light, humidity, changes in temperature, and wind shifts causing the aerosolized agent to be re-directed away from the target crop field [12].

Key agents for BW attacks against crops focus on the high calorie (i.e. carbohydrate) crops such as wheat, rice, corn, and potatoes [10]. Watson [28] and Whitby [10] describe some of these anti-crop agents: *Puccinia graminis* tritici (black stem rust), *T. indica* (karnal bunt of wheat), *Puccinia striiformis* var. *striiformis* (stripe rust of wheat), *M. oryzae* (rice blast), *C. miyabeanus* (brown spot in rice), *Xanthomonas oryzae* (rice leaf blight), *Puccinia Sorghi* (common rust of maize), *Cochliobolus heterostrophus* (southern corn leaf blight), *Xanthomonas vasicola* pv. Vasculorum (bacterial leaf streak of corn), and *Acidovorax avenae* subsp. *avenae* (corn bacterial leaf stripe), *P. infestants* (potato late blight), *Ralstonia solanacearum* (potato brown rot), *Streptomyces scabies*

(potato scab). Other crops for BW attack were chosen due to their international economic significance, such as coffee, bananas, citrus fruits, and sugar cane [10]. Some anti-crop agents for these crops include [10, 28, 29]: *Mycosphaerella musicola* (yellow sigatoka leaf spot of bananas), *Fusarium oxysporum F. cubense* (panama disease of banana), citrus tristeza virus (Tristeza of citrus), *Peronosclerospora sacchari* (downy mildew of sugar cane), *Sporisorium scitamineum* (sugar cane smut), *Colletotrichum kahawae* (coffee berry disease), *Liberibacter africanus* (citrus greening disease aka huanglongbing), and *Hemileia vastatrix* (coffee leaf rust).

16.5.2 Livestock Animals

The successful BW agents (like the anti-crop) agents need to be pathogens that can be easily cultured, easily stored until needed, easily disseminated, and have a high degree of virulence to the targeted population. Watson [28] described some of these agents: FMDV and rinderpest virus for cattle, Newcastle disease virus for poultry, *Ehrlichia ruminantium* for sheep and goats [30], and *Aspergillus fumigatus* for poultry.

Brown and Slenning discussed anti-animal diseases as a serious threat that could be introduced via smuggling infected animals into the country [31]. This would be one mode of attack that would not require aerial spraying of the pathogen, but could nonetheless introduce the pathogen in a coordinated BW attack or an act of bioterrorism. Furthermore, the authors note that if the anti-animal disease was introduced, it could remain endemic in the country as the pathogen could infect wildlife. One example of such an anti-animal disease is rinderpest which can infect both livestock animals, but also wild hoofed stock that exist in North America [31].

Gordon and Beck-Nielsen stated that foreign animal disease (FAD) pathogens could be key tools for a future bioterrorism attack against the livestock industry [32]. Besides the anti-animal pathogens mentioned above, the authors also include for FAD candidates avian influenza A virus and African swine fever virus (ASFV). ASFV could be a devastating anti-animal agent as this viral hemorrhagic fever can result in 100% lethality during the initial onset and no vaccine against the disease is available [32].

16.5.3 Bioterrorism Non-state Actors

Bioterrorists (aka non-state actors) might use agricultural BW in the following methods: multiple attacks with the pathogen at sites of high concentration of crops or livestock using contaminated animals (e.g. animals smuggled into the country with Avian influenza), pathogen aerosols (Karnal bunt teliospores for wheat crops or FMD in an aerosol for cattle), vectors carrying the pathogen (ticks carrying *E. ruminantium*) [32], or fruit bats or pigs (domesticated or feral) infected with Nipah virus [33, 34].

Jonathan Ban [2] discussed some of the reasons that BW against agricultural targets might be favored by bioterrorist. First, many of the pathogens are zoonotic and many do not affect humans, so without the risk of human fatalities, the moral restrains to using BW would be removed. Second, since the disease is agricultural, it may be very difficult to distinguish the attack from a natural outbreak of the disease. This is an important point, since many BW attacks (human or agricultural) would still require an incubation period from the time of exposure to the onset of disease. If the bioterrorist wants to avoid taking credit, the attack might be mistaken for a natural outbreak. Third, agricultural facilities and resources are largely exposed and not protected, hence very vulnerable to BW attacks. Fourth, the biotechnical (e.g. culturing a fungal plant pathogen) and operational barriers related to agricultural BW are relatively lower compared to human targeted BW weapons. Most agricultural BW will not infect humans (e.g. Karnal bunt, Late blight in potatoes, or FMD for cattle) and many will require a simple infection of a single animal or dispersal of a fungal pathogen over unprotected wheat or corn field [2, 29, 35–37].

O'Hara reported one unusual bioattack in 1997 that was initiated by pastoral farmers in the South Island of New Zealand [19, 36, 37]. The farmers introduced rabbit hemorrhagic disease virus (RHDV)-a calicivirus- on rabbit-infested farms to control the invasive rabbit population on the island. The farmers captured infected wild rabbits in Australia, homogenized rabbit tissues (liver and spleen) and applied the homogenized tissues to rabbit baits consisting of grain, carrots, and parsnips [36]. The farmers acted as the local bureaucrats did not act quickly in response to the farmers' demands for effective rabbit biocontrol. RHDV is now endemic in New Zealand.

16.5.4 Genetic Engineering

The risk of genetically engineered BW agents (aka Black Biology) is a key concern for agrobioterrorism [38]. Horn and Breeze [4] discussed this concern as a topic of growing potential weapons relevance. It is possible using genetic engineering techniques to enhance the toxicity or pathogenicity of organisms or toxins. It is also possible to engineer new organisms with enhanced capabilities to be resistant to antibiotics, vaccines, or to display a new series of symptoms [38]. The resultant BW agent directed at agricultural targets would create a greater vulnerability for any nation. Since without appropriate countermeasures (e.g., antibiotics, vaccines) or an extensive delay in disease identification (due to a new array of symptoms for the pathogen), any attack by a genetically engineered pathogen could potentially ruin the agricultural productivity of a nation.

16.6 Modes of Detection

The present strategy for BW agent detection includes diagnostic tools such as electrochemiluminescence, polymerase chain reaction (PCR) tied with enzyme immunoassays, and fluorogenic probe-based PCR [39]. Higgins et al described the feasibility of technical laboratory detection tools to be used in field-based laboratories [39]. The development of rapid diagnostics in a field laboratory was demonstrated by the 520th Theater Area Medical Laboratory (TAML) of the US Army. Higgins and colleagues state that the use of such rapid testing could provide rapid and accurate diagnosis of food borne or water borne BW agents. The authors also stress that rapid testing techniques would not be used alone; but would be used in conjunction with more traditional techniques to verify the pathogen and route to treatment [39].

Von Bredow et al. [26] describes several technologies developed for the detection of bacteria or contamination in food. One rapid method is a luminometer using a luciferin-luciferase reagent to detect live bacteria. The detection method has been found effective in detecting bacteria in either animal carcasses or in animal feed, even in samples of whole oats! Another rapid method of analysis and detection is the SMART (Sensitive Membrane Antigen Rapid Test) system developed by New Horizons Diagnostic Corporation. This test is an antigen-antibody two-step process that uses colloidal gold particles attached with the primary antibody to attach to the antigen (i.e. target BW agent). The system has been used to identify different BW agents, including *B. anthracis*, *Brucella melitensis*, *Francisella tularensis*, Botulism toxin, and Ricin. The authors state that the test can be modified for detection of other biological agents as soon as the appropriate antibody is developed [26].

Ron Sequeria of the USDA Animal and Plant Health Inspection Service (APHIS) noted that APHIS has expanded its capability to monitor BW attacks on agricultural facilities [39]. These strategies include use of geographic informational systems (GIS), Global Positioning Systems (GPS), satellite image analysis, remote sensing, and training an elite staff within an emergency response framework capable of managing the information processing and analysis. The APHIS framework can monitor the movement of epidemics and make necessary recommendations based on weather, geographic, and phytopathological data to halt or eradicate the epidemic. The author also noted that the APHIS activities will also be in close cooperation with other emergency management agencies as well as include cooperation from industry groups, state organizations, and academic institutions [39].

16.7 Vulnerability Factors of Agricultural Targets

Chalk [3] discussed the vulnerabilities of US agriculture to BW attacks and notes six primary vulnerabilities (Table 16.2).

First, the contemporary farming practices of concentrated and intensive farming practices. Ban [4] noted that 84% of the US cattle population is concentrated in the southwest, 60% of the swine population is located in the Midwest, and 78% of the

Table 16.2 Vulnerability factors of agricultural targets [3, 10, 12, 40]

1. Contemporary farming practices of concentrated and intensive farming practices
2. Increased susceptibility of livestock to disease
3. General lack of farm/food related security and surveillance
4. Inefficient and passive disease-reporting system exists that is further hampered by a lack of trust between regulators and producers
5. Most veterinarian's training does not include foreign animal diseases (or Biological Warfare diseases) and large-scale husbandry
6. Prevailing focus on aggregate, rather than individual, livestock health statistics
7. Monoculture as a farming practice becomes a large scale susceptible host to the pathogen infection and spread of the pathogen within the monoculture field
8. Low genetic variation (genetic uniformity) within agricultural crops and animals

chicken population is located in the southeast Atlantic region. Cattle are raised in feedlots holding as many as 150,000–300,000 head of beef, whereas chicken farms will pen 100,000 birds together [4]. This tight living arrangement allows for rapid spread of pathogens among livestock, especially if the pathogen is transmitted as an aerosol.

Second, the increased susceptibility of livestock to disease. Parker noted that intensive farming practices have stressed livestock weakening their resistance to disease. This results in an increased need for antibiotic use in feed stock and an increased risk of the development of antibiotic resistance strains of pathogens [6].

Third, a general lack of farm/food related security and surveillance [3]. Although this has been discussed previously, one example of the poor security is the cavalier attitudes of farm workers entering and leaving chicken pens. Bruce Stewart-Brown [41] reported a survey done at one large chicken farm where personnel freely entered and exited chicken pens. Many did not sign in or sign out, while few if any monitored what was tracked into the pens via shoes or clothing. Stewart-Brown noted that the lack of security practices and failure to prevent contamination via shoes or clothing could result in an easy and rapid transfer of pathogens from one large chicken pen (holding 100,000 or more chickens) to another. Furthermore, Stewart-Brown noted that outsiders entering the pens were not required to clean off shoes nor were required to provide any identification. Thus, outsiders visiting the farms could easily transfer pathogens from soil off of their shoes to various chicken pens and be active BW terrorists [41].

Fourth, Chalk mentioned that an inefficient and passive disease-reporting system exists that is further hampered by a lack of trust between regulators and producers. Chalk noted that the communication lines with state regulatory personnel are crude, and in many cases, confusing. Furthermore, farmers are reluctant to report disease outbreaks for fear of undergoing livestock "depopulation" (without compensation) in an effort to stop the outbreak of the disease [3]. This resistance to report and poor reporting communication systems engenders the conditions for rapid outbreaks and poor evidence chains to track back the disease to the source of the epidemic.

Fifth, Chalk discussed the problem that most veterinarian training does not include foreign animal diseases (or Biological Warfare diseases) and large-scale

husbandry [3]. Since large scale husbandry is the prevalent method of modern farming, most veterinarians fail in diagnosing diseases unique to large scale husbandry or in being able to detect conditions that are conducive for an epidemic. Also, since most veterinarians have not had training in foreign animal diseases (or biological weapon-based diseases); the opportunity to rapidly detect and stop an outbreak will be lost. This is not unusual since many younger physicians have not been trained in recognition of human directed (anti-personnel) BW, such as smallpox, anthrax, or Ebola [3].

The sixth vulnerability factor identified by Chalk [3] is a prevailing focus on aggregate, rather than individual, livestock statistics. Chalk described this factor as a result of large livestock populations. As farmers have such large populations of livestock, they tend to miss problems with individual animals and rather focus only on large scale results (e.g. total milk output). This large-scale data tends to miss individual animals that could be the incubator of a major outbreak of disease that would quickly spread throughout the crowded herd of livestock.

Two other factors have to be mentioned as vulnerability factors. Monoculture is the farming practice resulting in only one crop being raised in a field (e.g., wheat, corn, tomatoes, barley). As a result, the monoculture becomes a large scale susceptible host to the pathogen infection and spread of the pathogen within the monoculture field [6, 10, 12]. If the pathogen can spread beyond that field by airborne particles (such as fungal spores), then the pathogen can successfully spread to other fields or across the country or even across the continent. The spread by aerial dispersal of plant disease pathogens on a global or continental scale was described in great detail by Brown and Hovmeller [42]. The authors noted that long distance dispersals of fungal pathogen spores by the wind can spread plant diseases across or between continents. Furthermore, the irregular nature of these long-distance dispersals of fungal pathogens can create epidemics in new territories or create outbreaks in previously resistant plant cultivars [42]. This last observation could be a warning to those that consider using agricultural BW as the pathogens released in an enemy nation could blow back to the aggressor nation eventually with epidemic results.

The other vulnerability factor, is the low genetic variation (genetic uniformity) within agricultural crops and animals [12, 40]. Modern agricultural husbandry and plant genetics has resulted in reduced genetic variation within farm crops and livestock. With reduced genetic variation within livestock or crops, the potential for resistance to the pathogen is reduced. Furthermore, with low genetic variation within livestock and crops, the potential for finding genes for resistance is reduced as well [40].

16.8 Nonstandard Models of Attack

The following section will briefly discuss several possible routes of agricultural BW attack based on recent technological, economic, and scientific developments. These "nonstandard" models may become future attack models for agricultural BW in the twenty-first century.

16.8.1 Biocruise

Biocruise is defined as the combination of BW technology with cruise missile delivery systems. A cruise missile is defined as "an unmanned self-propelled guided vehicle that sustains flight through aerodynamic life for most of its flight path and whose primary mission is to place an ordnance or special payload on a target." [43]. This definition today includes unmanned air vehicles (UAVs) and remotely piloted helicopters or aircraft (RPVs). Cruise missiles are easier to obtain, maintain, and deploy than ballistic missiles. Cruise missiles have the advantage that a properly sized aerosol dispersal system could be installed within the missile. Once installed, the cruise missile could deliver a BW aerosol over a large swath area such as crop fields or livestock pastures or feedlots [43].

Some cruise missiles have extremely accurate navigation systems, using terrain contour matching (TERCOM) guidance systems, whereas others have guidance systems using the US Global Positioning System (GPS) or the Differential GPS (DGPS). With these systems, the accuracy of targeting by cruise missiles is far superior to ballistic missiles [43].

Kiziah [44] discussed the biocruise threat from the perspective that a biocruise attack could provide "plausible deniability" from a rouge nation. If the attack was done at night, a long-range land attack cruise missile (LACM) could be directed to disperse the BW agent while programmed to fly a circuitous route to the target. After dispersal, the missile could be programmed to crash in the ocean or self-destruct. Since cruise missiles fly low (some below radar detection level) and have a small Infrared (IR) and radar signature, detection of cruise missiles is difficult. Further, cruise missiles can be launched from sea (even launched covertly from a cargo or tanker ship), from the air, and from a submarine.

With biocruise technology, any nation or terrorist group could direct a cruise missile to navigate and disperse BW agents over agricultural targets; especially at night when notice by farmers or farm security is at the lowest level. With GPS navigation, the missile could disperse anti-crop or anti-animal agents over a number of targets, self-destruct in the ocean, and hence hide any evidence of a deliberate BW attack on agricultural resources.

Although it has not been described in detail; the application of drone (remotely controlled aerial vehicles) technology for shorter range delivery of agroterrorism agents, could evoke an agroterrorism attack of serious magnitude even if the bioterrorist was a "lone wolf".

16.8.2 Attacks Against Biofuel Crops

With the rise in demand for liquid fuels such as ethanol and biodiesel, biofuel crops like corn or sorghum for ethanol production and soybeans or palm oils for biodiesel production, will be prime targets for BW. Either a competing nation or a bioterrorist using BW to attack the crops could achieve a multiplier effect with an attack on corn,

soybeans, or sorghum: an epidemic on the crops, a resulting shortage of raw materials for biofuel production; and a subsequent shortage of biofuel.

16.8.3 Use of Introduced Species

An introduced species (aka exotic species) is a foreign organism introduced into an ecosystem and causing damage to that ecosystem [33, 45, 46]. Some organisms have been introduced and caused damage to agriculture (e.g. Kudzu and Gypsy moth in US, rinderpest in Africa, rabbits in Australia). Barnaby [40] discussed that the biodiversity of the planet is decreasing and this includes the genetic diversity of crop plants such as wheat and rice. One of the problems of emerging plant diseases is that some pathogens are "exotic" species until they have achieved establishment within new territories. Bandyopadhyay and Frederiksen [47] discussed the rise of some of these plant diseases as merely exotic species introduced into new habitats. These diseases include Sorghum ergot, Karnal bunt of wheat, Potato late blight, and *Citrus tristeza*. The authors further assert that these introductions can occur naturally or via trade practices [47].

If a nation or bioterrorist were to introduce a non-native pathogen to a susceptible agricultural target, it could have a devastating effect [33]. Hence, it would be possible to use the knowledge of the ecological success of introduced species to apply it as an agricultural BW weapon. One candidate for such application is Striga (aka Witch weed) [33]. Striga is a parasitic plant that consists of several species; all of them can grow underground and invade plant roots, robbing the host plant of water and nutrients [48]. The target host plants (depending on the species of Striga) are corn, rice, and sorghum. Originally from Africa where the parasitic plant is a menace, the plant was accidently introduced into the Carolinas, where plant quarantine was set up to contain the infestation [48]. At present, no complete eradication of Striga from the Carolinas has occurred.

16.9 Counterstrategies

Casagrande [35] has stated that biological attacks against agriculture should be regarded as a "high-consequence, high probability" event as a well as a grave "national security risk" [35]. Hence, counterstrategies should be given high priority in any discussion of agricultural BW.

16.9.1 Detection

Although the detection technologies presently have been described above, one key aspect in the detection is how to determine if the outbreak or epidemic was caused by natural means or by a BW attack [49].

Table 16.3 Indicators in the determination that an agricultural outbreak was intentional

1. Use of non-traditional pathways
2. Increase of the probability of survival of the pest in transit
3. Widespread dissemination of the disease from disparate foci
4. Use of highly virulent strains
5. High rates of inoculum
6. Introduction into remote areas
7. Targeting of susceptible production areas
8. Targeting of susceptible natural environments
9. Release of multiple species simultaneously
10. Precise timing of releases to coincide with maximal colonization potential

Note: intentional introductions differ from accidental introductions in the following means: Based on Sequeira [50]

Sequeira [50] described the following points to help in determining that the outbreak is intentional (Table 16.3).

This criterion is used for pathogens or for other "introduced species". Sequeira noted that intentional introductions will differ from accidental introductions in the following ways: (1) use of non-traditional pathways; (2) increase of the probability of survival of the pest in transit; (3) widespread dissemination of the disease from disparate foci; (4) use of highly virulent strains; (5) high rates of inoculum; (6) introduction into remote areas; (7) targeting of susceptible production areas; (8) targeting of susceptible natural environments; (9) release of multiple species simultaneously; (10) precise timing of releases to coincide with maximal colonization potential. Sequeira also noted that the globalization of the economy has already taxed the existing USDA structures and resources [50].

Rogers [51] noted that anti-crop BW has potential in nations where crop strains are susceptible to a pathogen. This risk is further enhanced if by genetic modification, a strain (or strains) of the pathogen are intended to affect the specific varieties of a crop grown in a target state. Rogers further noted that a state that is vulnerable to anti-crop BW, is a state with a system of arable agriculture which uses extensive monoculture of important crops; but lacks a well-developed research and extension service. The lack of monitoring, education, and research means that the state lacks the infrastructure necessary to rapidly fend off an agricultural BW attack (or a bioterrorist BW attack on agriculture) [51].

With regard to anti-animal BW attacks, Hugh-Jones [52] described some of the indicators as follows: the event has: unusual time and/or place of occurrence; unexpected strain of agent or multiple strains; a noted reversal of an otherwise steady progress in disease control or freedom; an epidemiologically "weird" event or occurrence that does not match normal experience or knowledge. Hugh-Jones notes that these events lead to the following results: marked economic or political costs with benefits going to a competitor; removal of the target country from international trade (quarantine); the target country must still continue imports from the competitor; there is marked social unrest in a significant part of the population

due in part to the loss of livestock or crops and jobs [52]. From these indicators, Hugh-Jones recommended steps to prepare for future incidents, assessing data to determine the suspicious outbreaks (including identification of the spread of the disease and the strain of the pathogen), analysis of economic and trade effects, determination of people movements of possible suspects involved in the incident; and finally publicity with reports properly detailing the known data for review by the scientific community and the public at large [52].

Furthermore, detection can be bolstered further by advanced training and tools for the farms and first responders [53] on plant and animal pathogens, such as introducing farmers [54], veterinarians [55, 56], and customs and border agents [55, 56] to enhanced training in exotic pathogens, phytopathology, and early detection systems for animal diseases. For example, Chomel and Marand [56] discussed the need for expanding coursework for veterinary students in wildlife zoonotic diseases, emerging diseases, and training in the reporting pathways for notifiable diseases in their country and state.

Knutsson et al. [57] described how biotraceability can enhance the response phase during a bioterrorism attack to feed or the food chain. The authors defined biotraceability as the ability to use downstream information to indicate the process or the specific food chain where the source of an agent (e.g., microbiological agent) was introduced. Thus, regardless of an accidental or deliberate pathogen or toxin entering the food chain, biotraceability techniques, biomarker tracer discovery, tracking tools, and communication reduce the response phase and enhance the tracing of the origin of the biological agent contamination [57].

16.9.2 Recommendations for Increased Cooperation and Communication Between Agricultural Agencies, Other Federal Agencies, and the Military

From the tools developed by the USDA, Sequeira reported that the USDA has accessed existing emergency response structures (including APHIS, Plant Protection and Quarantine (PPQ), and Veterinary Services (VS) of APHIS) and developed the formal organization, Regional Emergency Animal Disease Eradication Organization (READO). All of these organizations are directed to assist in the containment and eradication of pathogenic or introduced organisms resulting from a BW attack [50]. Casagrande [35] recommended that funding to APHIS should be increased to create Early Response Teams consisting of three-member teams that can respond to an animal or plant outbreak in 24 h.

In 1998, agricultural bioterrorism was not given proper attention under the Presidential Decision Directive 63 (PDD-63) which dealt with “Critical Infrastructure Protection”. PDD-63 did not list food and agriculture was one of the eight critical infrastructures that needed to be protected from Weapons of Mass Destruction (WMD). Although President Clinton did issue both PDD-63 and PDD-62 (PDD-62 dealt with “Combating Terrorism”) at the same time, agriculture was given a subcommittee under PDD-62 [6]. Parker described how the USDA should

be in the front of leadership in dealing with agricultural bioterrorism or BW attacks directed at agricultural resources. The USDA should lead in the bioterrorism strategy since its federal role is food safety and food security.

Parker concluded his book [6] with a series of recommendations, including: taking the lead in agricultural bioterrorism from the federal level; secure intelligence from the various intelligence agencies and maintain contacts with them; continue to cultivate a relationship with the military and use them where necessary in securing eradication efforts and maintaining order; expand contacts with state and local government agencies and academic institutions; develop partnerships with the private sector, especially with Farm Bureau Federation, national commodity organizations, and agribusiness organizations (e.g., American Poultry Association, National Cattlemen's Beef Association, National Corn Growers Association) and major agribusiness companies, feed companies, food wholesalers, slaughterhouses, seed companies, and other agribusiness related firms-large and small [6].

Martensson et al. [58] described a similar strategy of building networks of intelligence, police, forensics, customs agencies, along with the public and animal health and environmental organizations to share information to prevent an agroterrorism incident. AniBioThreat is a European Union project with a strategy of early warning and workshops to identify and build a collaborative culture to prepare and confront bioterrorism or agroterrorism threats [58].

In essence, communication with the public will help maintain order and help engender trust when an agricultural BW attack does surface.

Finally, Yeh et al. [54] discussed control and preventing acquisition of anti-livestock agents from laboratories and cell banks. Furthermore, the authors recommended that paperwork requirements for obtaining livestock pathogens be as stringent as obtaining human pathogens. Also, Yeh et al. [54] recommended that any request for livestock pathogens that seem unusual or suspicious be reported to government authorities with the case forwarded to a national investigative agency for possible review of bioterrorist activity.

16.9.3 Genetic Engineering

One tool that can be very useful for defense against agricultural BW is the exploration of genetic engineering. Genetic engineering of plants has led to major improvements in food quality and composition [59], but it has also provided new opportunities to improve insect resistance of the plants [60]. Dixon et al. [61] reported success in enhancing the natural defense responses of plants by boosting phytoalexin responses which can play a critical role in resistance to viral, fungal and bacterial pathogens. If this work expands and continues to be successful, it is possible that anti-crop pathogens could have their outbreaks blunted or blocked by crops with genetically engineered enhanced natural defenses. Finally, Gressel et al. reported success in the development of herbicide resistant plants which would allow use of herbicides to stop plant parasites like Striga and Broomrape; yet allow the target crop to flourish [62]. These techniques would offer opportunities for the

developing world to deal with parasitic plants, but could also provide tools to counteract parasitic plants that would be used as anti-crop BW.

16.9.4 Advanced Agricultural Techniques

Finally, advanced agricultural techniques will be required to break away from the modern agricultural methods that make present day agriculture so vulnerable to BW.

First, monoculture as a practice increases the vulnerability of the field crops to a BW attack. Intercropping with two different crops (e.g. rows of beans between rows of corn) would decrease the vulnerability of the whole field to a rapid spread of a pathogen. Next, many monoculture crops use an asexual means to propagate the plants (e.g., strawberry plants from stolons) which would reduce genetic diversity in the field. If all of the plants are genetically the same (asexually they are cloned from the "mother" plant), then this process would also contribute to increased vulnerability to a BW attack. Genetic variation within a crop field must be encouraged to reduce this risk.

Barnaby [40] commented that the genetic diversity of wheat and rice are becoming impoverished. Yet, it is the primitive cultivars that contribute to new genetic traits being bred into the germ lines of various food crops [40]. Barnaby recommended more intercropping practices, expanded work on integrated pest control and biological control agents, as well as development of resistant cultivars. With these improvements, crops would be more resistant to anti-crop BW attacks.

16.10 Summary

Agricultural products are a key part of US infrastructure, a major part of the US Gross Domestic Product, and a vital part of the US export trade. Agriculture and food has been taken for granted in the US due to the relative low cost, abundant productivity, and enhanced modern techniques for raising crops and livestock. Unfortunately, with these modern techniques, agriculture has become quite vulnerable to anti-crop and anti-animal BW. The reasons for the use of agricultural BW range from nations attacking overtly or covertly to destroy an enemy nation's food resources, to terrorist motivations for blackmail and extortion, evoking public fear, or profiting from commodity market turmoil following a BW attack on agricultural commodities.

History has demonstrated that many nations have explored or fully developed anti-crop and anti-animal BW. Although banned by the Biological Toxins and Weapons Convention (BTWC) treaty, agricultural BW may still exist in some nations and in the plans of bioterrorists (whether as a group or a lone disgruntled individual). As the food chain and food production techniques have become more complex, the vulnerability for agricultural BW has increased, both on the farm and in the food processing plant. Furthermore, food and water borne pathogens could be

used as BW agents to obtain the greatest number of victims through contaminated food or water.

At present, agricultural BW agent detection methods exist for a variety of agents. Furthermore, federal and state agencies have developed the necessary tools and protocols to contain, identify, decontaminate, and eradicate any agricultural BW agent. Improvements are necessary to reduce the response time to an attack (i.e., response phase) and enhance the cooperation of farmers, food producers, and the public in general. These improvements include having the USDA lead the response and communication with federal, state and local organizations in the event of an agricultural BW attack. Finally, the Federal government needs to recognize that agriculture is one of the critical infrastructures that must be protected from terrorist attacks.

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