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Strategies toward vaccines against *Burkholderia mallei* and *Burkholderia pseudomallei*

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Abstract

Burkholderia mallei and *Burkholderia pseudomallei* are Gram-negative, rod-shaped bacteria, and are the causative agents of the diseases glanders and melioidosis, respectively. These bacteria have been recognized as important pathogens for over 100 years, yet a relative dearth of available information exists regarding their virulence determinants and immunopathology. Infection with either of these bacteria presents with nonspecific symptoms and can be either acute or chronic, impeding rapid diagnosis. The lack of a vaccine for either bacterium also makes them potential candidates for bioweaponization. Together with their high rate of infectivity via aerosols and resistance to many common antibiotics, both bacteria have been classified as category B priority pathogens by the US NIH and US CDC, which has spurred a dramatic increase in interest in these microorganisms. Attempts have been made to develop vaccines for these infections, which would not only benefit military personnel, a group most likely to be targeted in an intentional release, but also individuals who may come in contact with glanders-infected animals or live in areas where melioidosis is endemic. This review highlights some recent attempts of vaccine development for these infections and the strategies used to improve the efficacy of vaccine approaches.

Keywords

bioweapon; *Burkholderia*; glanders; immunization; melioidosis; vaccine

Infections caused by *Burkholderia mallei* & *Burkholderia pseudomallei*

Burkholderia mallei and *Burkholderia pseudomallei* cause the diseases glanders and melioidosis, respectively. While these bacteria are phylogenetically similar, their lifestyles and epidemiology are quite divergent. Specifically, *B. mallei* is an obligate mammalian pathogen that typically infects solipeds, such as horses, mules and donkeys, and only occasionally infects humans. Individuals most at risk of contracting the disease are animal handlers in close contact with infected creatures and those who ingest contaminated meat. Glanders was effectively eradicated in North America and Western Europe in the 1950s by the mass culling of infected animals, but remains in the equine population of Africa, Asia, the Middle East and Central and

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South America. There have been no natural cases of glanders reported in the USA in over 60 years.

Conversely, *B. pseudomallei* is recognized as an important human pathogen endemic to Southeast Asia and Northern Australia, although it is not limited to these areas, since cases of melioidosis have been reported elsewhere [1–3]. The bacterium is an environmental saprophyte and can be cultured from wet soil and ground water. Humans most likely to contract the disease are those who have prolonged contact with contaminated water and soil, such as farmers and others exposed to the natural environment. Interestingly, outbreaks of melioidosis are subsequent to typhoon season and flooding in several endemic areas [4–6]. Farmers in this part of the world rarely wear protective footwear when harvesting rice and other crops that depend on these wet seasons; the feet of these individuals often show signs of repeated trauma and injury, which probably represents the route of infection [7]. Aerosols created by heavy rains can also increase the likelihood of inhalation of this pathogen. In addition, epidemiological studies suggest an inverse correlation between physical wellbeing and susceptibility to infection, since individuals with diabetes, compromised liver or decreased renal function appear to have increased risk of infection [8].

Glanders and melioidosis may present as either acute or chronic diseases, and there are no pathognomonic signs of infection, which may hinder prompt diagnosis. In an acute infection, general symptoms include fever, malaise, abscess formation, pneumonia and sepsis. Even with aggressive antibiotic therapy, septicemia caused by *B. pseudomallei* has a mortality rate of approximately 40% [9]. Since there has only been one documented case of human glanders in North America since 1949 [10], less is known about survival rates in individuals with *B. mallei* infections. Chronic melioidosis is often characterized by similar, albeit milder, symptoms than the acute disease and may last for months or even years [11]. Reactivation of chronic *B. pseudomallei* infections have occurred in Vietnam veterans up to 18 years after their last exposure to the bacteria, a condition nicknamed ‘the Vietnamese time bomb’ [12]. Reactivation is often correlated with the onset of other illnesses, such as influenza infection, Type 2 diabetes and even cancer [13]. *B. mallei* and *B. pseudomallei* can be contracted via abrasions in the skin and/or inhalation; the dose and route of infection probably determines the severity of symptoms that develop.

B. mallei and *B. pseudomallei* are facultative intracellular bacteria, capable of infecting a wide range of cell types [14]. This fact may help explain the long periods of latency observed in some infections. It is likely that intracellular replication and survival may also provide the bacteria with a means for evading the humoral immune system. Such factors should be taken into account when developing possible vaccine strategies.

The high rate of infectivity via aerosols, their resistance to many common antibiotics and the absence of a vaccine for either infection make these bacteria of great concern as modern bioterror agents. Indeed, *B. mallei* is a proven bioweapon that was used in both World Wars I and II. It has been suggested that the former Soviet military used this agent more recently in Afghanistan and that they were also weaponizing *B. pseudomallei* [15]. With respect to natural infection with *B. pseudomallei*, even when the infection is treated early and aggressively with antibiotics, melioidosis has a high rate of relapse [11]. More recent studies suggest that approximately a quarter of those relapses may actually be reinfection with another strain [16], or that the initial infection was caused by multiple strains [17]. An effective vaccine against these pathogens would not only protect those at high risk for natural infection, such as those in countries where the microorganisms are endemic, but could also reduce the desire to weaponize and intentionally release these bacteria.

Genomic analysis of *B. mallei* & *B. pseudomallei*

Although these bacteria have been relatively understudied, the recently sequenced genomes of *B. mallei* and *B. pseudomallei* have fostered the development of genetic tools for the determination of virulence factors, and allowed for the comparison of these two pathogens at a genetic level. Sequence analyses have revealed remarkable similarities and differences between the two species. Of note, the genome of the sequenced *B. mallei* strain ATCC 23344 [18] is smaller than that of the sequenced *B. pseudomallei* strain K96243 [19] (5.8 compared with 7.25 Mb, respectively). When these genomes were compared with one another, more than 1400 genes were either absent or variant in *B. mallei*. However, genes common to both species were highly homologous and organized similarly along the genome. Amino acid identities of predicted homologs between these two organisms were greater than 96%. Full alignment of the two genomes revealed over 80% identity, and predicted proteins had high mean values of identity (98.8%) and length match (99.7%) [20]. The majority of genes absent in the *B. mallei* genome were clustered on the *B. pseudomallei* genome and the deletion of these genes is consistent with insertion element-mediated mechanisms. As such, *B. mallei* is widely regarded as a niche-specific deletion derivative of *B. pseudomallei*.

Strikingly, there are relatively few *B. mallei*-specific genes [20], which suggests that a vaccine effective against *B. mallei* may also be effective against *B. pseudomallei*. More recently, the genomes of *B. mallei* and *B. pseudomallei* were compared with five non-pathogenic but closely related *Burkholderia* spp. *In silico* genomic subtraction identified 650 genes common to both pathogens but absent in the nonpathogenic strains, indicating that the products of these genes are putative virulence determinants and possible crossprotective vaccine targets [21].

Interestingly, even within the type strain of *B. mallei* (ATCC 23344), genetic variation can be observed. This strain was investigated for genomic stability when passaged through a variety of mammalian hosts [22]. The majority of observed changes occurred within intergenic regions; however, some mutated coding sequences resulted in altered protein expression. The observed genome instability was often due to changes in the number of repeating units within ‘simple sequence repeats’. Another report recently described the attenuation of a previously virulent strain of *B. mallei*, SAVP1, upon passage of the bacterium in a cohort of equids [23]. Sequencing of this attenuated strain revealed the loss of a number of virulence-associated genes, notably those encoding the type III secretion system required for virulence in both hamsters and mice. As such, the authors make the argument that this strain should be considered a candidate for exclusion from Select Agent Regulations [23]. If accepted, this may encourage increased research on this pathogen in the USA, since experiments would not need to be performed in biosafety level (BSL)-3 facilities.

While similar passage experiments have yet to be published for *B. pseudomallei*, it has been documented that morphotypic variation exists among clinical clones in *B. pseudomallei* infection [24]. In this study, particular morphotypes were associated with extremely high mortality when transferred to BALB/c mice, while other morphotypes were far less fatal and appeared to display chronic infection phenotypes. This implies that morphotype switching may be correlated with an increase in the production of factors associated with *in vivo* concealment [24]. The genomic plasticity of these two pathogens suggests that host adaptation and/or immune evasion may alter gene expression, and should be considered during the development of therapeutics against these two pathogens.

Animal models of glanders & melioidosis

Identifying a relevant infection model is critical for defining the virulence of a particular pathogen and key for the advance of efficacious vaccines against that microorganism. TABLE 1 summarizes the animal models that have been described for *B. mallei* and/or *B.*

pseudomallei as well as their significance for vaccine development. Those anticipated to be of greatest clinical relevance will be discussed in more detail.

One of the most common models used for the study of *B. pseudomallei* and, more recently, *B. mallei* pathogenesis is the Syrian golden hamster [25,26]. These animals are extremely susceptible to virulent *B. pseudomallei* and *B. mallei* infection, while less virulent strains require a 4–5-log increase in inoculum to achieve equivalent mortality. Thus, the hamster provides an elegant model by which the virulence of particular strains may be assessed. Where this model falls short, however, is in the exquisite sensitivity of these animals to the pathogens, which does not accurately mimic human infection. Therefore, a model slightly more resistant to infection with these organisms may provide a more physiologically relevant system for vaccine development.

As with other infectious agents, inbred mice represent powerful tools for the study of both virulence and immunity, and both BALB/c and C57BL/6 mice have been characterized as relevant infection models for melioidosis. Experiments performed by Leakey *et al.* described in detail the differential infection outcomes of *B. pseudomallei* in these two mouse strains [27]. A 4-log difference in the lethal dose that kills 50% of subjects (LD_{50}) was measured between these two strains of mice when bacteria were administered intravenously, with BALB/c mice being more sensitive than C57BL/6 mice. In addition, BALB/c mice appeared to mirror acute melioidosis, unlike infection in C57BL/6 mice that mimicked the chronic course of infection. While only BALB/c mice presented with bacteremia, both BALB/c and C57BL/6 mice showed significant bacterial colonization in the liver and spleen. Recently, it was discovered that BALB/c and C57BL/6 mice develop differential inflammatory responses as a result of infection with aerosolized *B. pseudomallei* [28]. Specifically, the production of proinflammatory cytokines was markedly higher in BALB/c compared with C57BL/6 mice. It was concluded that hyperproduction of proinflammatory cytokines, specifically IFN-γ, in the BALB/c mice was not protective, and may even lead to septic shock in this model. By contrast, the C57BL/6 mice reacted to infection with a moderate and transient induction of proinflammatory cytokines, which enabled them to clear the infection at the given dosage [28], a result consistent with observations in outbred mice, where administration of antibodies to IFN-γ increased susceptibility to *B. pseudomallei* infection [29]. The finding that BALB/c and C57BL/6 mice respond in a dissimilar immunological fashion to *B. pseudomallei* infection may account for their differences in susceptibility, and may refect similar differences between susceptible versus nonsusceptible individuals.

The BALB/c mouse model of infection ha s also been used for the study of glanders pathogenesis. The organotropism present in melioidosis infection is also present in glanders infection in this model system, as bacteria localize specifically to the liver and spleen. Of particular biodefense interest is the fact that these mice appear to be more susceptible to *B. mallei* by the aerosol route than the intraperitoneal route [30]. This route of infection is also clinically relevant, since inhalation of *B. mallei* is one of the major routes of human disease, making it an attractive model in which to study the efficacy of potential vaccine strategies.

Until very recently, little was documented concerning experimental equine glanders. For *B. mallei* infection, solipeds are certainly the most physiologically appropriate model, since they represent the natural reservoir for this bacterium, although other animals have been noted as harboring the disease as well [31]. In 2003, Lopez *et al.* systematically described the clinical presentation of glanders in horses intratracheally inoculated with *B. mallei* [32]. Infected animals developed inflammatory nodules and ulcers in the nasal cavity, with increased sticky, yellow nasal secretions, and enlarged and firm submaxillary lymph nodes. The horses also exhibited progressive debility, febrile episodes and dyspnea [32]. Interestingly, necropsy of these animals revealed that nasal mucosa was reliably positive for *B. mallei* colonies, but

bacterial dissemination to other organs was rare. Although working with the equine model of glanders is extremely challenging and expensive, the use of this biologically relevant model may prove valuable for the future of vaccine research against *B. mallei*.

Vaccine approaches for *B. mallei* & *B. pseudomallei*

As mentioned previously, these facultative intracellular bacteria are capable of residing in the vacuoles of eukaryotic cells, which can complicate both antibiotic treatment and vaccine development [30,33]. It is believed that intracellular survival is a key virulence determinant for both of these microorganisms. Therefore, it is likely that a cell-mediated immune response, perhaps in addition to a humoral response, may be critical for protection, as has been shown for other intracellular bacterial pathogens [34].

A number of virulence factors have been recognized in *B. mallei* and, in most cases, have also been identified as relevant in *B. pseudomallei* virulence [35]. TABLE 2 outlines some of the antigens of these two pathogenic agents that have been exploited for the development of vaccines, as well as immunotherapy treatments used in an attempt to thwart the diseases caused by these bacteria. While TABLE 2 is not meant to be an exhaustive list, it does highlight some of the more promising vaccine candidates against these two pathogens, detailing the routes of administration and challenge. For reasons described previously, the overwhelming majority of these vaccines were tested in the BALB/c mouse model of infection. A notable exception to this is a protein polysaccharide conjugate vaccine mentioned in the study describing the equine model of glanders [32]. To our knowledge, none of the vaccine procedures outlined have progressed to clinical trials. Antigens and therapies of particular interest will be discussed further.

Killed, whole-cell vaccines

Nonviable, whole-cell bacterial preparations have traditionally represented a starting point in the development of vaccines, and these have been shown to be effective in the prevention of chorea, typhoid fever, whooping cough, anthrax, and plague [36]. A similar approach was employed by Amemiya *et al.* in 2002 against *B. mallei* in BALB/c mice using three separate preparations of the bacteria: heat-killed, irradiated and an irradiated capsule-negative mutant [37]. It was reported that each vaccine preparation yielded similar immune responses when splenocyte activation and sera immuno-globulins were analyzed. Splenocytes expressed a variety of cytokines, such as IFN-γ, IL-4 and IL-10, which indicated that there was a mixed reaction to the vaccine and that mice were unable to generate a directed Th1- or Th2-like response. In addition, antibody titers in the sera of these mice suggested a Th2 skew, as the relative level of IgG_1 was markedly higher than the level of IgG_{2a}. The authors saw no protection at very high doses (>300 × LD_{50}) when mice were challenged intraperitoneally [37]. However, a more recent paper from the same laboratory showed that the addition of IL-12 to a vaccine preparation of irradiated *B. mallei* was able to preferentially enhance the amount of IgG_{2a} generated in the sera, thereby inducing a more Th1-like antibody response [38]. IL-12 also induced an increase in the proliferation of splenocytes as well as the amount of IFN-γ produced by these cells when compared with mice vaccinated with killed *B. mallei* alone. Ultimately, mice vaccinated with IL-12 and *B. mallei* were better protected against a high challenge dose (>100 × LD_{50}) 21 days after infection when compared with mice that received either killed *B. mallei* or IL-12 alone. However, the spleens of the vaccinated survivors were greatly enlarged and heavily infected with *B. mallei*. These results suggest that the Th1-like response, induced by the addition of IL-12 to the preparation, improved the efficacy of this vaccine strategy in the context of BALB/c infection.

Live-attenuated vaccines

The efficacy of attenuated live bacteria as a protective vaccine has been tested for both *B. mallei* and *B. pseudomallei*. In 2005, Ulrich *et al.* studied the ability of two differently attenuated strains of *B. mallei* (a capsule-negative mutant and a branched-chain amino acid auxotroph), delivered aerogenically, to protect against aerosolized *B. mallei* challenge [39]. Serum samples from vaccinated mice revealed that animals generated a Th2-like antibody response to the capsule-negative mutant, with substantially higher titers of IgG_1 compared with IgG_{2a}. It was determined that this immune response was not protective, as no mice challenged with aerosolized *B. mallei* survived 5 days after infection. Conversely, the immune response to the auxotrophic mutant was skewed toward a Th1-like antibody response with a high IgG_{2a}-to-IgG_1 ratio. As a result, 50% of these mice were able to survive high (>300 × LD_{50}) aerosolized challenge. When the spleens of these mice were analyzed 30 days later, the authors found more than 10^5 *B. mallei* cells in every mouse, indicating that although the mice were able to survive the lethal challenge, they were not able to completely clear the infection [39].

Similar results were obtained when a homologous *B. pseudomallei* auxotroph [40] was investigated as a protective vaccine against *B. pseudomallei* infection [41]. However, these investigators delved further into the mechanism of partial protection. Splenocytes from vaccinated mice proliferated *in vitro* in response to whole nonviable *B. pseudomallei*, and $CD4^+$ and $CD8^+$ T cells responded by increasing the production of IFN-γ, consistent with previous reports underlining the importance of IFN-γ in protection [29,42,43]. To determine the roles that specific T cells play in protection with regards to this vaccine, immunized mice were depleted of either $CD4^+$ or $CD8^+$ T cells before and after *B. pseudomallei* challenge. Immunized $CD4^+$ T-cell-depleted mice were substantially more susceptible to infection compared with immunized mice given isotype control antibodies, and succumbed to infection at the same time as unimmunized control mice, indicating that protection was abrogated with the loss of $CD4^+$ T cells. By contrast, $CD8^+$ T-cell depletion had no effect on vaccine-mediated protection. Taken together, these data demonstrate that protection generated by this vaccine is mediated by $CD4^+$ T cells, but not by $CD8^+$ T cells [41].

Polysaccharide-based vaccines

Bacterial polysaccharides are often potent stimulators of host immune responses and represent critical components of subunit and conjugate vaccines for clinically relevant diseases, such as *Haemophilus influenzae* type b, pneumococcal and meningococcal infections. Lipopolysaccharide (LPS) has been shown to be an immunodominant antigen recognized in patients infected with *B. pseudomallei*. Importantly, the level of antibody to LPS on admission to the hospital was higher in patients with melioidosis who survived compared with those that died, and in patients with nonsepticemic versus septicemic melioidosis [7], suggesting that these antibodies may protect the host from death. Alternatively, it may be the case that the presence of high anti-LPS antibody titers is indicative of a more efficient host immune response, including cell-mediated killing [7].

Importantly, the LPS of *B. pseudomallei* and *B. mallei* are remarkably similar. The O antigen portion of LPS from each species is composed of a disaccharide: [→3)-β-D-glucose-(1→3)-6-deoxy-α-L-talose-(1→]$_n$. Where these two O antigens differ however, is in the location and level of *O*-acetyl substitutions on the talose residue. None of the O antigen polysaccharide structures from *B. mallei* LPS are acetylated at *O*-4 and are variably acetylated or methylated at *O*-2, while *B. pseudomallei* produces two structures – one that is acetylated at *O*-4 and partially methylated (33%) at *O*-2, and a second that is not acetylated at *O*-4 and is partially acetylated (67%) at *O*-2 [44,45].

B. mallei and *B. pseudomallei* are encapsulated bacteria whose capsular polysaccharides have been shown to be important virulence determinants in Syrian golden hamsters and BALB/c mice [46,47]. The structure of the major capsular polysaccharide of *B. pseudomallei* was determined as [-3)-2-*O*-acetyl-6-deoxy-β-D-*manno*-heptopyranose-(1-] by Perry *et al.*, but it was characterized at the time as type I *O*-polysaccharide. However, Reickseilder *et al.* later identified this structure as the capsular polysaccharide [46,47]. The chemical structure of *B. mallei* capsular polysaccharide has yet to be determined but, based on gene homology and organization, DeShazer *et al.* suggested that the polysaccharide is similar, if not identical, to the major capsule structure of *B. pseudomallei* [48]. Both *B. mallei* and *B. pseudomallei* have additional surface polysaccharides that have been recently described in another review [49]. Taking into account the genetic and structural similarity that exists between characterized polysaccharides of these species, it is possible that a vaccine specific for either the LPS and/or the capsule of *B. mallei* or *B. pseudomallei* may be able to protect against both pathogens.

Both the LPS and capsular polysaccharides have been evaluated as subunit vaccine candidates against *B. pseudomallei* by Nelson *et al.* in 2004 [50]. In this study, it was discovered that BALB/c mice responded differently to the two surface polysaccharides. Specifically, mice intraperitoneally immunized with LPS generated high titers of IgM and IgG_3, which were augmented with the addition of adjuvant. On the other hand, mice immunized with capsular polysaccharide, presented with an IgG_{2b} response. When adjuvant was added to the capsule preparation, an increase in IgM was measured but no increase in any other antibody subtype was reported [50]. Vaccination with either subunit was able to increase the mean time to death in mice when compared with unimmunized controls (2.6 days for unimmunized, 10.5 days for capsule and 17.6 days for LPS) when challenged intraperitoneally at 250 × LD_{50}. The authors saw a negligible increase in protection when mice were challenged with lower doses (2.5 × LD_{50}) via the aerosol route [50]. Unfortunately, as with all other *B. mallei* and *B. pseudomallei* vaccination protocols that we have seen in the literature to date, protection engendered by this procedure was not complete.

Recognizing the importance of LPS-specific antibodies in patients with melioidosis, passive immunization with monoclonal antibodies for *B. mallei* LPS has been evaluated for vaccine efficacy against glanders infection [51]. In this study, three different LPS-specific monoclonal antibodies were able to significantly protect BALB/c mice from death up to 14 days postchallenge with a high aerosolized dose of *B. mallei* (20 × LD_{50}). These results represent the first successful immunotherapeutic protection against the bioweaponization-relevant administration of *B. mallei*. This suggests that these antibodies provide relevant protection against this pathogen in the initial stage of infection. However, the anti-LPS antibodies were not able to provide protection when administered 18 h after *B. mallei* infection [51], suggesting that at that time point, the bacteria had been internalized and were therefore resistant to the circulating antibodies. Alternatively, during this later stage of the infectious process, *B. mallei* may not be expressing the specific epitope to which the antibodies are directed. In any case, a lack of effiective antibodies after infection allowed the bacteria to spread and cause disease.

Cell-mediated immunity

The results of the various vaccination strategies outlined in this review signify that a cell-mediated immune response, in conjunction with a humoral response, is probably required to successfully clear infections caused by *B. mallei* and *B. pseudomallei*. However, the cellular immune response needed to clear these infections is proving to be intricate and complex. Perhaps surprisingly, Haque *et al.* demonstrated that $CD8^+$ cells were dispensable in protection when mice were vaccinated with an auxotrophic mutant of *B. pseudomallei*. However, the precise level of attenuation of this mutant has yet to be defined. It is possible that these bacteria

are less able to invade host cells to cause disease, which would lessen the capacity to generate a protective cytotoxic T-cell response with this vaccine in this particular model. In addition, since vaccinated mice were unable to successfully clear challenge infection in this study, even in the presence of both $CD4^+$ and $CD8^+$ cells, the potential remains for the necessity of cytotoxic T lymphocytes in the clearance of host cells infected with *B. pseudomallei* [52]. Amemiya *et al.* were able to highlight the importance of both IL-12 and IFN-γ in the promotion of an effiective immune response against *B. mallei*. This is consistent with the finding that melioidosis patients routinely present with elevated serum levels of IFN-γ and IL-12 [53]. It is widely accepted that IFN-γ production is the hallmark of Th1 cells; natural killer and $CD8^+$ T cells are also known to produce this cytokine. Several of the studies previously outlined above also indicate that a skewed Th1-like antibody response increases survival in mice infected with either *B. mallei* or *B. pseudomallei*. However, Tan *et al*. demonstrated that brute force expression of IFN-γ is likely to contribute to the immunopathogenesis of *B. pseudomallei* in BALB/c mice [28], underlining the importance of a balanced and regulated immune response in the clearance of this pathogen. Ultimately, the exploitation of the differential immune responses generated by BALB/c and C57BL/6 mice during experimental infection may provide additional clues about the proper immune response needed to effectively eradicate these infections, and may provide the nidus for the next generation of vaccine development against these lethal pathogens.

Expert commentary

Glanders is an ancient malady of horses that was described by Hippocrates around 425 BC and by Aristotle in 330 BC. The importance of this disease is exemplified by its use as a bioweapon in several wars. In fact, *B. mallei* is the only pathogen on the Select Agent List that is known to be used deliberately as a military weapon. Melioidosis was first identified by Captain Alfred Whitmore, a British pathologist serving in Burma around 1911. At that time, the disease was described as a glanders-like illness in opium addicts. The prevalence and distribution of infection by *B. pseudomallei* appears to be increasing: recognition of melioidosis increased sharply in Thailand with the increase in hospital microbiology facilities able to identify this infection [11]. More recent identification of *B. pseudomallei* infections outside Southeast Asia and Northern Australia suggests that these bacteria may have a wider range of endemicity than was previously appreciated [1–3].

Although glanders and melioidosis have been recognized for nearly 100 years (or more), relatively little has been described about the biology of the bacteria that cause these illnesses. Recently, there has been a resurgent interest in these pathogens, mainly due to their characterization as potential bioterror agents; as such, there has been a strong focus on vaccine development. Genome sequencing has provided the springboard for new insights into the relatedness of these microorganisms and their potential virulence factors and vaccine candidates, yet many questions remain. In particular, a clearer understanding of the complexity of the lifestyle of these bacteria, their survival in the host and environment and the natural history of infection and disease are topics of study that have only been recently addressed, a fact that underlines the need for more research into the basic biology of both of these pathogens.

The facultative intracellular lifestyle of *B. mallei* and *B. pseudomallei* makes infections by these bacteria difficult to treat and means they require an extended course of treatment with multiple antibiotics. The survival within host cells and the results of the various vaccine regimens against these microorganisms suggests that a cell-mediated immune response, in addition to a humoral response, will be necessary for complete protection from *B. mallei* and *B. pseudomallei* infection. The genomic similarity of these organisms suggests that vaccines developed against one of these bacteria may be suitable for clearance of the other. Aside from those living where these pathogens are endemic, a vaccine against these bacteria would be

particularly significant for military personnel and emergency medical workers, who would be the first to respond in the event of an intentional release.

Five-year view

The future of *B. mallei* and *B. pseudomallei* vaccinology should be focused on a better understanding of the natural history of the infectious process in both animal models and the human host. The route of infection and the dose obviously impact the course of the diseases caused by these pathogens. Futhermore, there are many bacterial factors that are responsible for virulence. The finding that *B. mallei* has lost segments of its genome and, in doing so, has limited its host range to equids and other mammals, suggests the importance of these factors for survival in the natural environment. On the other hand, the facultative intracellular nature of both of these bacteria suggests common mechanisms of survival in the host environment.

There also appear to be host factors responsible for susceptibility that need further investigation. The use of the BALB/c versus C57BL/6 mice to model susceptibility and the acute versus chronic infectious process is an appropriate start; however, future studies will need to be performed as these mice are distinctly different in many respects. Genetic crosses between these mouse strains may begin to identify loci responsible for infection resistance or susceptibility. In addition, immunization and protection studies in mice with specific innate and adaptive immune defects should continue to define the host factors responsible for resistance.

The requirement for authorized BSL-3 facilities and trained personnel to perform studies on these pathogens in the USA has hampered rapid progress on vaccine development. The related bacterium *Burkholderia thailandensis*, which occupies the same environmental niches as *B. pseudomallei* [54], has been suggested as a model organism by some investigators. However, others have questioned its relevance since it generally does not cause overt disease in humans and the LD_{50} in hamsters and mice is up to 10^6-fold higher than that of *B. pseudomallei*. Alternatively, if avirulent derivatives of these bacteria could be considered exempt from select agent status, special secure laboratories would not be required to work with these organisms and, as such, vaccine development would be greatly accelerated.

Vaccinology is generally considered an empirical science. However, the recent advancements in genomic sequence analyses has allowed for the identification and prioritization of potential vaccine candidates. The field of ‘reverse vaccinology’ makes use of bioinformatic analysis to identify surface-associated proteins that may represent appropriate vaccine targets [55]. Antigens can also be further evaluated *in silico* by T-cell epitope-mapping tools to identify those that may shift responses to a Th1 phenotype. An additional global approach for antigen detection utilizes ‘protein microarrays’. This high-throughput technique can rapidly convert genome sequence information into proteins, which are then purified and applied to glass slides. Sera from infected animals or humans can be added to the slide and immunoreactive antigens can be detected [56]. The application of these technologies to the study of *B. mallei* and *B. pseudomallei* would augment the selection of potentially viable vaccine targets that could be evaluated for immunogenicity and protection in the available animal models of infection. Once identified, such candidates could be further developed for testing in human clinical trials.

Key issues

- *Burkholderia mallei* and *Burkholderia pseudomallei*, the causative agents of glanders and melioidosis, respectively, are understudied. They are highly virulent bacteria that have been developed as bioweapons.

- The bacteria are facultative intracellular pathogens and there are no vaccines available to either of these microorganisms.
- The recent classification of these as priority pathogens has increased the interest of the scientific community in these select agents.
- Infections in BALB/c and C57BL/6 mice mimic the acute and chronic diseases, respectively.
- Genomic analyses have revealed that these bacteria are closely related, with *B. mallei* being a niche-specific deletion-derivative of *B. pseudomallei*.
- The similarity of these pathogens suggests that one vaccine candidate may be effective against both agents.
- Vaccination using mouse models of infection have suggested the importance of a cell-mediated, as well as a humoral, response for protective immunity.
- Future studies using comparative genomics and reverse vaccinology, as well as techniques for detecting cell-mediated immune responses, will be critical for the generation of effective vaccines, which would probably diminish the desire to weaponize these potentially lethal pathogens.

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Papers of special note have been highlighted as:

• of interest

•• of considerable interest

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Table 1

Animal models of *Burkholderia mallei* and *Burkholderia pseudomallei* infections.

Animal model	Pros	Cons	Ref.
Syrian golden hamster	Well characterized, cost effective	Extremely sensitive (LD_{50} < 10 CFU)	[25,26]
C57BL/6 mouse	Isogenic, well characterized, multiple routes of infection	Chronic infection specific	[27]
BALB/c mouse	Isogenic, well characterized, multiple routes of infection	Acute infection specific	[27,31]
Horse	Clinically relevant	Cost prohibitive	[32]
Diabetic rat	Clinically relevant	*Burkholderia pseudomallei* tested, only infant rats are susceptible	[57]
Pig	Clinically relevant	*B. pseudomallei* tested, chronic infection specific, high infectious dose	[58]
Galleria mellonella (wax moth)	Cost effective, rapid screening of virulence factors	Physiological relevance to clinical infection unknown	[21]
Caenorhabditis elegans	Cost effective, rapid screening of virulence factors	Limited sensitivity due to high infectious dose, may be *B. pseudomallei* specific, not physiologically relevant	[59,60]

CFU: Colony-forming unit; LD_{50}: Lethal dose that kills 50% of subjects.

Table 2

Antigens, immunotherapies and other treatments used as vaccines against *Burkholderia mallei* and *Burkholderia pseudomallei*.

	Bacteria targeted	Route of administration	Challenge method	Ref.
Antigens				
Killed whole cell	*Burkholderia mallei*	sc.	ip.	[37]
Attenuated live whole cell	*B. mallei* and *B. pseudomallei*	Aerogenic, ip., in.	Aerosol, ip., ip.	[39,41,61]
LPS	*B. pseudomallei*	ip.	ip. or in.	[50,62]
CP	*B. pseudomallei*	ip.	ip. or in.	[50,62]
Pili	*B. mallei*	sc.	Aerosol	[63]
Outer membrane proteins	*B. pseudomallei*	ip.	ip.	[64]
Type III secretion system subunits	*B. pseudomallei*	ip.	ip.	[65]
Immunotherapies and other treatments				
IL-12 with killed whole cells	*B. mallei*	sc.	ip.	[38]
Monoclonal antibodies to LPS	*B. mallei*	ip.	Aerosol	[51,66]
Monoclonal antibody cocktail to LPS, CP and proteins	*B. pseudomallei*	iv.	ip.	[51,66]
CpG oligodeoxynucleotide	*B. mallei*	ip.	Aerosol	[67]
Primed dentritic cells with CpG	*B. pseudomallei*	id.	ip.	[68]
DNA encoding fagellin	*B. pseudomallei*	im.	iv.	[69]

CP: Capsular polysaccharide; id.: Intradermal; im.: Intramuscular; in.: Intranasal; ip.: Intraperitoneal; iv.: Intravenous; LPS: Lipopolysaccharide; sc.: Subcutaneous.