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Animal Models for Melioidosis

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Abstract
Purpose of Review Development, testing, and evaluation of medical countermeasures for melioidosis are hampered by a lack of well-characterized and standardized animal models. Recent work has both refined existing animal models for this disease and identified new ones.
Recent Findings Head-to-head comparisons of mouse strains with varying susceptibility to the organism and using different routes of infection highlighted and confirmed important similarities and differences between murine models and exposure routes. Diabetic mouse models provided insight into the disease process in humans having this major risk factor. Large animal models, both livestock and non-human primate, have been established. Alternative (non-mammalian) models have been useful in identification of virulence factors and screening of therapeutic candidates. They hold potential for large-scale screening that would not be appropriate or practical for mammalian species.
Summary Recent advances in animal and alternative modeling will enhance our understanding of the organism and the disease process, as well as accelerating the development of medical countermeasures.

Keywords Melioidosis · Animal models · In vitro models · Medical countermeasures · Virulence · *Burkholderia pseudomallei*

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Introduction

Burkholderia pseudomallei is a normal soil and water saprophyte predominately found in Southeast Asia and Northern Australia and the causative agent of the disease melioidosis in these regions and elsewhere, including Africa and the Americas [1, 2]. Melioidosis is considered to be an emerging disease, in part due to improved diagnosis. To a lesser extent, it is also due to migration and transport of infected animals [3–7] and increased immigration from and tourism to endemic regions [8–10], allowing for latent infected hosts to transport the disease to non-endemic regions. Transmission of this disease is thought to be by various routes: oral, respiratory, or breaks in the skin [11–15]. Melioidosis outbreaks have also been reported in a wide variety of animals, including both terrestrial and aquatic mammals, reptiles, birds, and fish in various settings (natural habitat, domestic, and zoological as reviewed in [16–19]). For domestic animals, natural infection is most commonly reported in sheep, goats, and swine [16, 17, 20].

The U.S. Department of Health and Human Services categorizes *B. pseudomallei* as a tier 1 biological select agent. *B. pseudomallei* is thought to pose a threat for use as a biological weapon because (1) hosts are susceptible to infection via aerosol, (2) a long latency period is possible prior to the development of clinical signs, (3) the organism has intrinsic resistance to many antibiotics, and (4) no vaccine is currently available. As such, the biodefense community has taken much interest in developing appropriate animal models for developing medical countermeasures (MCM) for melioidosis. Here, we provide a review of the diverse animal models available for melioidosis that provide a path forward for development of medical countermeasures and future pre-clinical studies.

Mouse Models

Mouse models have been extensively used to characterize the pathogenesis of *B. pseudomallei*. Data have illustrated that BALB/c mice are more susceptible and may represent an acute model of melioidosis; whereas, C57BL/6 mice are significantly more resistant and may represent a more chronic model of disease. While there is debate about the definition of chronicity in a mouse model, clear differences in susceptibilities are observed in these mice after challenge with *B. pseudomallei.* Accordingly, work on these two strains constitutes the majority of data available for *B. pseudomallei* mouse models. Obvious benefits of working with mice include the relatively low cost, ease associated with animal husbandry, and the ability to attain statistical significance with larger animal numbers. One drawback may be the route-associated sensitivities with some of the mouse strains. For example, some strains have a median lethal dose (LD_{50}) of less than 1 colony-forming unit (CFU) when delivered as an aerosolized dose. There are also obvious issues associated with the application of data obtained from mouse experiments to human disease [21, 22]. Table 1 offers a quick reference guide to what we consider important or noteworthy studies designed to characterize the mouse model of melioidosis [24–30, 31•, 32]. Due to space constraints, we have focused mainly on mice exposed to aerosolized bacteria or mice that received intraperitoneal (IP) inoculations. However, other routes have been examined including intravenous (IV), intranasal (IN), and subcutaneous (SC).

Intraperitoneal Exposure The IP route of exposure has been used as an efficient way for characterizing pathogenesis and ranking the virulence of *B. pseudomallei* clinical isolates. The IP route offers a potential bridge of exposure routes between laboratories for testing as it is easy to perform and there are potentially less variables than when delivering aerosolized bacteria. Welkos et al. demonstrated the IP route of exposure was able to clearly discern differences in virulence associated with a panel of clinical isolates. This report detailed LD_{50} values calculated for day 21 and day 60 after exposure to the bacteria [30]. Because of the extended endpoint, the importance of observing infected mice for extended periods of time in order to accurately assess the final outcome of the infection was demonstrated. This study has important consequences on the design of vaccine and therapeutic studies, particularly in the choice of bacterial strains and experimental endpoints. Additionally, Welkos et al. provided evidence suggesting an inverse correlation between in vitro and in vivo virulence of *B. pseuudomallei* as determined within the parameters of the macrophage cell model employed. It was shown that strains of *B. pseudomallei* more cytotoxic to in vitro to the J774.A1 tissue culture cells were generally less virulent in the mouse model [30]. While further data are required to substantiate this observation, this inverse correlation may model an important aspect of intracellular *B. pseudomallei* biology. Other reports have also used the IP model of inoculation (see Table 1), and the cumulative data demonstrate that this model is a valuable tool for *B. pseudomallei* research. While IP challenge is obviously not a natural route of infection, the resulting disease progression presents with interesting pathology and clinical signs which can be anecdotally compared to human case reports. Some examples described in the literature include pyogranuloma formation in various organs [33, 34], primary pneumonia [9] and secondary pneumonia after parenteral inoculation [35], and orbital cellulitis or necrotizing fasciitis [36].

Aerosol Exposure Given that *B. pseudomallei* is transmitted via exposure to aerosolized bacteria during monsoon seasons in endemic areas and also that *B. pseudomallei* is a HHS tier 1 agent of concern for biological defense, the aerosol route of exposure is of significant importance [37–39]. Several reports have detailed this route of exposure using both C57BL/6 and BALB/c mice and further characterized the course of disease and importance of the biodefense-driven animal model [25, 26, 29, 31•, 32]. More recent reports have demonstrated that variation between differentially virulent bacterial strains can be ascertained in an aerosol model [29]. However, whereas the IP model offers a relatively wide range of LD_{50} values between clinical isolates [30, 40], the differences observed in LD_{50} values when mice are exposed to aerosolized *B. pseudomallei* are much more subtle. Due to the difficulty in delivering reproducibly small doses of bacteria and the findings revealing a narrower range of virulence differences between clinical isolates, this mouse model is technically challenging and limited to facilities with specialized equipment.

Exposure to aerosolized bacteria results in a primary pneumonia that leads to a highly disseminated disease course in mice (and humans) [9, 29, 31•, 32, 34]. The pathologies observed after exposure to aerosolized bacteria have some similarities to those seen after IP inoculation, but the variety and severity of these observations are different than the IP route [31•]. For example, caudal disease progression (e.g., rear end paralysis or tail lesions) can be observed after mice inhale *B. pseudomallei* but is less commonly observed and may be less severe when contrasted with similar observations in mice receiving the bacteria via an IP injection [30, 31•].

These differences are hypothesized to be due to the initial site of entry and associated with local draining lymph nodes. Even with these important discussion points and caveats, the mouse model offers a highly useful system in which to study bacterial pathogenesis as well as test and evaluate critically needed novel MCMs.

Table 1 Noteworthy mouse modeling studies for melioidosis

	Mouse strain(s)	Route	Bacterial strain(s)	Key data and experimental findings presented
Dannenberg and Scott 1957 [23]	Albino Namru	IP Aero	103-67 (mouse adapted)	1. Long time course was examined, up to day 80 in some cases 2. Ranges of doses employed (from 2 to 100,000 LD_{50} equivalents) 3. Examined histopathology and gross pathology 4. Descriptive immune cell population data presented 5. Compared chronic disease in mice with humans
Leakey et al. 1998 [24]	BALB/c C57BL/6	IV IP	Clinical isolate from fatal case in 1990	1. Provided evidence for differential model in C57BL/6 mice compared to BALB/c mice 2. Modeled mainly early disease: most data are 96 h time course with mention of later times 3. Performed Mendelian analyses with generations of BALB/c and C57BL/6 mice 4. Bacterial burden provided for select tissues (blood, liver, spleen) 5. Examined growth kinetics in peritoneal exudate cultures from BALB/c and C57BL/6 mice; demonstrated that C57BL/6 cells were significantly more microbicidal than BALB/c cells
Tan et al. 2008 [25]	BALB/c C57BL/6	IP Aero IN SC	K96243	1. Modeling acute disease: 6 day time course was examined with 3 time points 2. Cytokine/chemokine expression was reported for sera and lung samples 3. Bacterial burden provided for select tissues (nasal wash, blood, lung, spleen, liver) 4. Confirmed different susceptibilities of mouse strains 5. Differential immune responses of the mouse strains: high levels of pro-inflammatory cytokines were detrimental and contributed to the immuno-pathogenesis
Lever et al. 2009 [26]	BALB/c	Aero	BRI	1. Modeled acute disease: through 120 h with moderate dose of bacteria (20 LD_{50} equivalents) 2. Bacterial burden provided for select tissues (lung, liver, spleen, blood, kidney, and brain) 3. Histopathology 4. Confirmed the sensitivity of the BALB/c mouse to melioidosis
Srisurat et al. 2010 [27]	BALB/c	IP	A2	1. Exposed mice to high dose (230 CFU) or low dose (6 CFU) to characterize chronicity 2. Moderate time course was reported, up to day 28 in some cases 3. Bacterial burden provided for select tissues (blood, spleen, liver, lung) 4. ELISA data were presented describing resulting antibody responses
Conejero et al. 2011 [28]	C57BL/6	IN	576	1. Long time course was examined, up to day 90 in some cases 2. Bacterial burden provided for select tissues (blood, liver, spleen) 3. Cytokine/chemokine expression was reported for sera and lung 4. Histopathology and gross pathology 5. Provided evidence that a chronic mouse model of melioidosis may be similar to that of humans
Massey et al. 2014 [29]	BALB/c	Aero	K96243; HBPUB-10303a	1. Achieved low delivered aerosol dose (5.4 and 3.8 cfu) 2. Data were collected for a short to moderate time frame up to 14 days post exposure 3. Blood chemistry and hematology parameters were examined 4. Cytokine/chemokine expression was reported for sera and lung samples 5. Bacterial burden provided for select tissues (lung, liver, spleen) 6. Examined histopathology 7. Temperatures and weights were recorded daily 8. Underscored the importance of standardization of models

Table 1 (continued)

	Mouse strain(s)	Route	Bacterial strain(s)	Key data and experimental findings presented
Welkos et al. 2015 [30]	BALB/c	IP	K96243; 1026b; 1106a; 406e; MSHR5858; MSHR5848; MSHR5855; MSHR305; MSHR668; HBPUB-1034a; HBPUB-10303a	1. Long time course was examined, up to day 90 in some cases 2. Bacterial burden provided for select tissues (blood, lung, spleen and liver) 3. Cytokine/chemokine expression was reported for sera and spleen 4. Characterized immune cell populations with flow cytometry 5. Histopathology and gross pathology 6. Established the utility of the IP model for ranking virulence of clinical isolates based upon LD_{50} 7. Presented data suggesting an inverse correlation between in vitro cytotoxicity and in vivo virulence in the cell model employed
Bearss et al. 2017 [31•]	BALB/c C57BL/6	IP Aero	K96243	1. Long time course was examined, up to day 90 in some cases 2. Bacterial burden provided for select tissues (blood, lung, spleen) 3. Cytokine/chemokine expression was reported for sera and spleen 4. Characterized immune cell populations with flow cytometry 5. Histopathology 6. Delivered purposefully low doses and similar LD_{50} equivalent doses 7. Comprehensive description of a common lab strain in both mouse strains using 2 routes 8. Documented multi-nucleated giant cell (MNGC) formation in mice following infection

IP intraperitoneal, *IV* intravenous, *IN* intranasal, *SC* subcutaneous, *Aero* aerosol

Diabetic Mouse Models Epidemiological studies have consistently identified diabetes as a major risk factor, with 39–57% of patients being diabetic or diagnosed with diabetes at presentation [41–43]. Only a limited number of studies have been conducted with either type 1 diabetic (T1D) or T2D diabetic animal models of melioidosis. T2D models are preferable as they represent 90–95% of all diabetes patients, which also reflects the melioidosis patient population [44]. The earliest models were T1D models induced by streptozotocin treatment in rats or mice [45–50]. In general, the studies reported significant differences between diabetic and normal mice in organ burden, cytokine levels, or gene expression, but none reported significant differences in mortality. Most recently, a model of melioidosis in Akita mice, which have a point mutation in the Ins2 gene leading to β-cell apoptosis, has shown a significant difference (> 2.5 Log_{10} CFU) in the LD_{50} between diabetic and control animals. It is a T1D model, but characteristics of the T2D phenotype, such as insulin resistance and changes in cardiac structure and function, are present [51].

Type 2 diabetic models have included polygenic/diet-induced and leptin-receptor knockout mouse models [52, 53]. The leptin-receptor knockout (db/db) is the only model that has shown a statistically significant difference in mortality. However, the db/db model is not representative of human T2D in either pathogenesis or severity. The diet-induced diabetic models are believed to be more representative of human diabetes. Relative to control animals, they have shown decreased expression of inflammatory cytokine RNAs, increased inflammatory cell infiltrates, and higher organ burdens early in infection [52, 53]. All of the above diabetic studies (T1D and T2D) have been conducted in male mice and rats since the females are resistant to develop the diabetic phenotype. This contrasts with virtually all other experimental models of melioidosis, which use only females. The diet-induced diabetic models are also restricted to the C57BL/6 strains (or hybrids). The importance of dietary composition for these models, particularly the fat to sugar/carbohydrate ratio and glycemic index, must be carefully considered. More work is required to generate an appropriate diabetic mouse model of melioidosis.

Hamsters and Guinea Pigs

The Syrian golden hamster, *Mesocricetus auratus*, has been utilized since the 1940s as an acute model of experimental melioidosis [23, 54–57]. Male and female hamsters are exquisitely sensitive and uniformly susceptible to infection with *B. pseudomallei*. The LD_{50} is typically less than 10 CFU and death occurs 2–5 days post-challenge.

Multiple routes of infection have been utilized, including IP, SC, respiratory, and oral, with little difference in disease progression. Melioidosis in hamsters is an acute fulminating infection with considerable morbidity by 48 h, often with a purulent ocular exudate. Recent studies by Gutierrez and Warawa demonstrated that the body temperature of hamsters infected with *B. pseudomallei* spikes approximately 24 h after infection and then drops rapidly immediately prior to death or euthanasia [56]. Systemic infection ultimately results in hamsters that harbor 10^3–10^8 CFU in the blood, liver, spleen, and lungs at disease endpoints.

The intrinsic sensitivity of hamsters to experimental melioidosis has been exploited by researchers to isolate *B. pseudomallei* from environmental sources [55, 58], characterize non-pathogenic near-neighbor species [54, 59], and identify integral virulence determinants such as the capsular polysaccharide, cluster 1 type VI secretion system (T6SS-1), cluster 3 type III secretion system (T3SS-3), quorum sensing, phospholipase C (PlcN3), and a two-component system sensor kinase (BPSL2025) [60–64]. Tuanyok et al. utilized a whole-genome microarray to compare the RNA expression profile of bacteria growing in infected hamster organs with bacteria grown in vitro and found numerous differentially regulated *B. pseudomallei* genes important for in vivo metabolism and virulence [62].

Hamsters have also been used as an acute model of infection to confirm the attenuation of *B. pseudomallei* strains subject to removal from the Federal Select Agent Program's select agent list. The strict rules and regulations associated with select agent research apply to *B. pseudomallei*, and there is a desire by the melioidosis community for strains with attenuated virulence that can be excluded from select agent requirements [56, 65, 66].

Guinea pigs have been used historically to detect and assess strain virulence of *B. pseudomallei* [57, 67–69]; however, their recent use as an animal model for melioidosis has been limited. The reported susceptibility of guinea pigs following challenge has varied between being acute (SC and IP) with all of the challenged animal succumbing to infection within a week [70] versus moderately susceptible with a great deal of in variability of survival following IP, SC, ingestion, and inhalational challenge [57]. In a study by DeShazer et al. [71], the LD_{50} for guinea pigs with strain 1026b by the IP route was 2000 CFU.

Large Animal Models

The study of melioidosis in non-rodent, vertebrate models can be broken down into two broad categories: livestock animals and non-human primates (NHP). These models are used much less frequently and have significant limitations relative to their rodent counterparts in terms of biocontainment housing, but have the advantage of being natural host models and, in the case of NHP, having more similarity to humans. Despite the very broad host range of *B. pseudomallei* [16–18, 72], studies in wild rodents rarely found evidence of disease or even seropositivity in rats or mice [73, 74]. In contrast, melioidosis has been well-described in goats, sheep, and other livestock [20, 75–89], which parallels disease in humans with the exception that most livestock tends toward chronic presentations of melioidosis with granulomatous lesions [90]. Natural disease in NHPs has been reported as well, but less frequently [3, 4, 7, 91]. Of the species used for experimental models, natural disease (outside of zoological gardens) has only been reported for two rhesus macaques [18]. Both exhibited chronic or reactivated latent disease, with diagnosis 6 months and 10 years after acquisition [3, 91].

Livestock Experimental infection of livestock has been conducted in chickens (IM) [92], horses (SC) [93], cattle (SC) [76, 94], pigs (IV and intratracheal) [95, 96], sheep (SC, IM, IV, supraconjunctival, IN, and oral) [76, 83, 84], and goats (SC, IP, and aerosol) [97–100]. The only species with recent efforts toward the development of livestock models of melioidosis are pigs and goats.

Natural infection in pigs is typically chronic or asymptomatic (discovered only at slaughter) and likely follows oral infection [20, 76, 80, 86, 87, 101]. The pattern of disease after experimental infection generally follows the pattern seen in other species, with a febrile response and multiple organ involvement, but a clear affinity for the lungs and spleen [95, 96]. Pigs appear to be resistant to acute disease in experimental infection, even with immunosuppression, and additionally appear capable of clearing the infection [93, 95, 96]. Pigs do not appear to be well-suited for the study of naturally occurring human melioidosis or biodefense-related countermeasure assessment.

The goat, as a naturally affected species, has a comparatively large base of the literature describing the presentation and lesions of caprine melioidosis [20, 78–80, 82, 97–99, 102], which compares well with human disease in terms of clinical presentation, epizootiology [16], organ distribution, and histopathology [34, 103]. Acute presentations are possible in goats, but chronic disease is more common and may in some instances self-cure with sterile lesions observed [77, 79, 98, 100]. Experimental infection of goats has been used for developing serodiagnostics (first description of indirect hemagglutination assay), examining the risk of mastitic goats' milk as a source of zoonotic infection, and characterizing disease pathogenesis as a model of human disease [97–100, 104, 105].

Recent aerosol and percutaneous infection studies in goats have provided detailed characterization of the pathology, histopathology, and pulmonary radiographic changes

associated with caprine melioidosis [99, 100]. Clinical signs of fever and the development of pyogranulomatous lesions in the lungs and spleen were typical of what is seen in humans and experimental models. Bi- to multinucleate giant cells were observed in pulmonary pyogranulomas following percutaneous infection [100], which have been reported from human cases [34, 103, 106], but they are relatively infrequently reported from animal infection models [31•].

Livestock models and reports of natural infection provide a valuable insight and comparative study to the field of human melioidosis. The ability of livestock to typically develop chronic disease, and in some cases self-cure, raises interesting ideas of examining different, but successful immune responses to melioidosis. However, the rarity of large animal biocontainment facilities capable of housing livestock coupled with limited immunologic reagents is significant barriers to use of these models.

Non-human Primate Non-human primate (NHP) models are considered the closest approximation to human disease given the close phylogenetic relation and comparable pathologic findings. However, an extremely wide range of susceptibility amongst NHP species exists [4, 57, 107]. Early investigations by Stanton and Fletcher and Miller et al. examined inhalation/nasal instillation, inoculation, scarification, intraperitoneal, or oral routes of infection, showed cynomolgus macaques (*Macaca fascicularis*) to be generally resistant to infection [57, 93, 108], even though outbreaks of melioidosis have been seen in imported cynomolgus macaques [4]. Recent NHP models development efforts have focused primarily on aerosol exposure models in the common marmoset (*Callithrix jacchus*), rhesus macaque (*Macaca mulatta*), and African green monkey (*Chlorocebus aethiops*) [109, 110, 111•, 112, 113]. The focus on aerogenic disease is driven by biodefense imperatives, though the importance of natural inhalational infection [38, 114–116] is supported to a similar extent as percutaneous infection [116–119] by epidemiological data.

The marmoset model employs a lower order NHP that is safer and easier to handle in biocontainment conditions compared to old-world monkeys, while still retaining immunologic and physiologic similarities to humans [111•]. Marmosets are extremely susceptible to *B. pseudomallei* with LD_{50}s < 10 CFU and death within 57 h when exposed to 10^2 CFU by aerosol [109]. Subcutaneous challenge similarly produced uniformly fatal disease within 85 h [112]. Disease is severe, acute, and consistent with fever, high levels of bacteremia, and typical lesions in the lung, spleen, and liver. However, lung lesions were notably absent in two thirds of animals infected subcutaneously [109, 112]. Despite the high susceptibility to *B. pseudomallei*, the marmoset model was able to show differential virulence amongst bacterial strains [111•]. However, factors such as the challenge dose remain more important in determining disease outcome or time to death [111•]. The greatest limitation to the marmoset model is how exquisitely sensitive is to *B. pseudomallei* with very low lethal doses and short times to death. By comparison, primary cutaneous melioidosis in humans is rarely associated with severe disease, whereas even low doses in marmosets produce fatal disease [112, 120]. This model would represent a very stringent test for therapeutic antimicrobials and is likely too severe for melioidosis vaccine testing.

A comparison of the natural history of inhalation melioidosis (aerosol dose ~ 3500 CFU) in rhesus macaques (RM) and African green monkeys (AGM) demonstrated that both species developed an infection that closely resembles that observed in acute human disease, including fever, leukocytosis, neutrophilia, anorexia, and dyspnea. The AGM uniformly developed a rapidly fatal acute disease and were more likely to have systemic involvement. Although 8/10 RM succumbed to acute disease, two survived in the study. One of these had symptoms of chronic pneumonia. Since the AGM was a more consistent model at this dose of *B. pseudomallei*, it was suggested that the AGM is a more appropriate model for MCM evaluation [110].

Alternative (Non-mammal) Models

Surrogate hosts have been explored as alternatives for *B. pseudomallei* challenges to reduce and replace mammals (when possible) for identifying bacterial virulence factors and testing therapeutic candidates. For initial characterization, these alterative models may prove beneficial as they could allow for large-scale screenings which would not be ethically appropriate or practical for mammal species. Furthermore, many of these surrogates have simple growth conditions, short generation times, less ethical and regulatory concerns, and reduced costs and space constraints for performing studies in biocontainment laboratories.

Nematode (*Caenorhabditis elegans*) The nematode *C. elegans* is a well-developed surrogate animal model for bacterial pathogenesis studies, and it shares many similarities to the mammalian innate immune system [121, 122]. The initial study on *B. pseudomallei* and nematodes demonstrated that this infection was an active process [123]. Further development of this surrogate model has identified additional *B. pseudomallei* virulence factors [124, 125] and potential therapeutics [126–128].

Madagascar Hissing Cockroach (*Gromphadorhina portentosa*) Insects have also proven to be a popular alternative model to mammals as there is a high degree of similarity in the innate immune systems [129]. Recently, the Madagascar hissing cockroach was tested for validity to serve

as a surrogate model for *B. pseudomallei* infection. Hemocytes from infected cockroaches appeared to provide an intracellular niche for the bacteria and to also form multinucleated giant cells. The cockroaches were highly susceptible to infection with the LD_{50} calculated to be < 10 CFU. In addition, when comparing LD_{50} measurements with several *B. pseudomallei* mutant strains, a clear correlation in virulence was observed between challenged cockroaches and hamsters [130]. A recent study demonstrated the therapeutic potential of the antimalarial drug chloroquine in treating *B. pseudomallei*-infected cockroaches [131].

Wax Moth (*Galleria mellonella*) An additional insect which has been examined as an alternative model is the larvae of the wax moth, *G. mellonella.* The wax moth larvae have been shown to be susceptible to infection with *B. pseudomallei* and able to support growth within the hemocoel [132]. Additional studies have examined *B. pseudomallei* strains at differing levels of virulence for mice and showed similarities to results obtained with macrophage-like cells and wax moth larvae [133].

Amoeba (*Dictyostelium discoideum*) *B. pseudomallei* is an intracellular pathogen that is also able to interact with amoeba. Due to the many similarities between amoeba and macrophages, *D. discoideum* has been explored as a model system using plaque assays to study intracellular survival of *B. pseudodmallei* [134]. This system may allow greater insight into the mechanisms of virulence of *B. pseudomallei* through studies of intracellular survival and replication.

Animal Models Applied to Pathogenesis and MCM

Animal models have been used to identify virulence factors (potential targets for antimicrobial and vaccine development), screen prospective vaccines, and therapeutics and evaluate the immune response to *B. pseudomallei* infection. The advanced development of a promising MCM for human use generally requires testing in a NHP model of disease, especially where controlled studies in humans are not ethically feasible. Only the most promising candidates are typically tested in NHPs due to cost and animal welfare considerations, and only a few melioidosis MCMs have been tested in NHPs [135, 136]. Thus, lower rodent and invertebrate animal models are invaluable resources. Their convenience and similarity in part to the human disease encourages their use in screening and development of novel candidate MCMs.

Numerous studies have been published over the past few years describing the use of BALB/c and C57BL/6 mice to identify novel *B. pseudomallei* virulence determinants and characterize the relative pathogenicity of clinical and laboratory-passaged isolates [137–145, 146•, 147]. Strain competition studies in the mouse model of melioidosis allow the identification and characterization of bacterial genes with mildly attenuated phenotypes [145]. Tn-seq transposon libraries identified 548 genes required for lung colonization and three large gene clusters, capsule, T3SS-3, and T6SS-1, responsible for much of the lung pathogenesis in the intubation-mediated intratracheal (IMIT) murine model of infection [139]. In addition to shedding light on mechanisms of pathogenesis, these types of analyses may aid in the identification of new bacterial targets for the development of MCM.

Recent reviews are available which describe and evaluate candidate melioidosis vaccines and established or novel therapeutics in animal models [148, 149, 150••, 151, 152–158]. The majority of this work has been done in murine models. Examples of novel therapeutics include new antibiotics, antimicrobial peptides, plant-derived compounds, herbicides, natural toxins/venoms, oligodeoxynucleotides (ODNs), and monoclonal antibodies (MAbs) [127, 159–168]. New regimens for current antibiotics have also been studied. For example, Ruiz et al. reformulated ceftazidime as a dry powder for efficient aerosol delivery to the lungs [169]. In addition to antibacterial treatments, therapeutics that target host pathways involved in responses to infection, or target and stimulate immune cells, such as T cells, have been characterized in vivo [136, 170–174].

Although some post-exposure therapeutics appeared to prevent *B. pseudomallei* infection [175], most animals ultimately become colonized and succumb to disease. Thus, vaccine-induced active immunity may be a more promising strategy for preventing infection. Several antigens have been reported to stimulate immunity against infection during the study course [148–154]. Most of these vaccines targeted surface antigens (LPS, capsule, outer membrane proteins, and killed whole cells). Recently, new formulations or platforms for known vaccine targets have provided protection, such as antigen-carrier glycoconjugates, micro- and nano-particle vaccines, outer membrane vesicles, and killed *B. pseudomallei* whole cells inactivated by alternate means [135, 176–183]. Alternately, live attenuated *B. pseudomallei* strains with auxotrophic or virulence factor gene deletions which are significantly attenuating, and recombinant *Salmonella* and *B. thailandensis* strains, have been leveraged as live vaccines, with the hope that they will generate more long-term, sterilizing immunity [184–189].

An understanding of the host response to *B. pseudomallei* is critical to MCM development. Although care must be taken in extrapolating immune responses in animal models to human disease, some recent studies have addressed changes in cytokine/chemokine levels in response to the organism [29, 190], the roll of Toll-like receptors (TLRs) in infection [191–202], and the importance of neutrophils in innate immunity [203, 204]. Comparison of mouse strains known

to differ in susceptibility to *B. pseudomallei*, such as BALB/c and C57BL/6, suggests that there is an early overt expression of inflammatory cytokines, such as IL-1α, IL-1β, IL-6, and TNFα, in the BALB/c mouse compared to that seen in the more resistant C57BL/6 mouse. Similarly, comparing cellular and humoral immune responses in a susceptible NHP host to those of a more resistant one could prove informative. Specific-pathogen-free and genetically modified (knockouts or humanized) mice are available and can be particularly useful for evaluating the immune response and the host-pathogen relationship.

Finally, animal models can be used for other purposes which indirectly support MCM development. These studies (1) spurred the development of select agent exempt stains which facilitate non-containment biosafety laboratory studies on MCMs [184, 205–208], (2) assessed the efficacy of current consensus antibiotic treatment regimens [175, 209–211], (3) probed the role of antibiotic tolerant in vivo variants in treatment failures [212–214], and (4) identified bacterial and host markers which might serve as correlates of protection and immunity [152, 172, 174, 215•, 216, 217]. For instance, a conventional post-exposure treatment regimen failed to eradicate infection in BALB/c mice under conditions of immunosuppression or upon extended persistent infection [209]. Also, in a treated mouse model, Cummings et al. characterized transcriptional responses of *B. pseudomallei* specific to ceftazidime exposure to identify markers of treatment efficacy [215•]. Further, long-term and rigorous studies (in mice and then NHPs) to identify countermeasures with the most promise for eradicating infection or producing sterile immunity are required to support the advanced development of human MCM candidates for melioidosis.

Conclusions

Recently, significant advances have been made in the development of animal models for melioidoisis. These include traditional (rodent), large animal, and alternative models. Head-to-head comparisons of mouse strains with varying susceptibility to the organism, and using different routes of infection, highlighted and confirmed important similarities and differences between murine models and exposure routes. Although diabetic mouse models are relatively early in development, they are providing some understanding of the disease process in the segment of the human population having this major risk factor for melioidosis. Promising large animal models include the goat, a naturally affected species, which compares well with human disease in terms of clinical presentation, organ distribution, and histopathology. However, it requires a biocontainment facility designed for livestock. NHP models, generally considered to be the closest approximation for human disease, vary in susceptibility to *B. pseudomallei* with the marmoset being exquisitely sensitive, and the AGM being intermediate between the marmoset and the Rhesus macaque. The choice of NHP may vary depending on the goal of the study, for example, testing of therapeutics versus vaccines. Alternative models, such as nematodes, amoebae, and insects, have been primarily useful in the identification of virulence factors and, to a lesser extent, screening of therapeutic candidates. They hold potential for large-scale screening that would not be appropriate or practical for mammalian species. All of these models will contribute to the development of MCM and a better understanding of melioidosis.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent Animal research at the United States Army of Medical Research Institute of Infectious Diseases was conducted and approved under an Institutional Animal Care and Use Committee in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

Disclaimers Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

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- •• Of major importance

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