DISSERTATION

THE DEVELOPMENT AND CHARACTERIZATION OF CAPRINE INFECTION MODELS OF MELIOIDOSIS

Submitted by

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ABSTRACT

THE DEVELOPMENT AND CHARACTERIZATION OF CAPRINE INFECTION MODELS OF MELIOIDOSIS

Melioidosis, the disease resulting from infection with *Burkholderia pseudomallei*, is a serious emerging infectious disease endemic to Southeast Asia and Northern Australasia and a leading infectious cause of death in the former. Additionally, *B. pseudomallei* has been designated a Category B Select Agent by the United States Centers for Disease Control and Prevention because of its potential use in bioterrorism, which has led to intensive research on inhalational models of murine melioidosis. Natural infection is believed to occur predominantly through percutaneous inoculation or inhalation in the rainy season in endemic areas, with infection following oral exposure occurring to a lesser extent. However, the actual importance of each route of infection in natural disease is unknown. Studies examining the comparative pathogenesis of melioidosis in regards to the route of infection are generally lacking, particularly in naturally affected species.

A goat model was selected as it provides the opportunity to study the importance of the route of infection and its effect on disease pathogenesis in a naturally affected species. Disease and outcome can be evaluated relative to natural presentations in both human and goat populations as goats and humans exhibit a similar epizootiology/epidemiology of melioidosis, which corresponds to similar environmental exposure to *B. pseudomallei* within its endemic range. Furthermore, the larger body size of goats allows for human-relevant clinical monitoring as well as longer-term serial evaluation of disease progression and therapy in individual animals.

Using a caprine model system, we have investigated the pathogenesis of infection following intratracheal aerosol and percutaneous exposure to 10^4 delivered colony forming units (CFU) of *B. pseudomallei*. Disease was observed in all animals following infection. Acute disease was more severe in aerosol infected goats, but both groups tended to develop subacute to chronic active disease, with percutaneously infected goats showing regression of lesions at the later time points. Percutaneously infected goats generally exhibited more variable clinical signs, hematologic changes, and gross pathology, but often had histologic lesions with more severe changes. Dissemination from the site of infection was much more rapid in the percutaneously infected animals, with bacteria detectable in the lungs and spleen as early as Day 2 post-infection (PI) and gross abscessation evident in distant sites as early as Day 7 PI. Extrapulmonary dissemination after aerosol infection appeared to occur around Day 7 with splenic or renal abscesses not grossly detectable until day 14. Lesion development was closely associated with a leukocytoclastic vasculitis observed in affected tissues in both aerosol and percutaneous infection. Pulmonary involvement was evident in all but one percutaneously infected goat (Day 2 PI) by culture or the presence of histologic lesions. The rapid dissemination of *B. pseudomallei* after percutaneous inoculation challenges the perception that inhalational melioidosis is more severe in presentation or will affect the lungs more frequently than percutaneous infection.

The findings presented here provide a detailed clinical, radiographic, and pathologic description of the pathogenesis of subacute to chronic aerosol and percutaneous caprine melioidosis. However, acute presentations are possible in association with concurrent disease or debility, suggesting that the caprine model system may be amenable to the incorporation of risk factors to increase susceptibility/acute disease as typically seen in human melioidosis.

It is hoped these models will help broaden the scope of melioidosis research to fill remaining voids, particularly in the areas immunology, vaccine development, and evaluation of novel antimicrobial therapeutics through the comparative study of disease. Additionally, melioidosis in goats remains a current problem in the regions of Southeast Asia and Northern Australia with enzootic and epizootic disease causing economic losses. Any knowledge gained from the use of these models in regards to improved diagnosis, preventive measures, and vaccination could be directly applicable to the management of these goat populations and advancing public health.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Melioidosis was first described as a glanders-like condition of morphine addicts and wastrels in Rangoon over 100 years ago [1], but it still plagues Southeast Asia and Northern Australasia as an emerging infectious disease. For a little over a decade, it has also been recognized as a potential global threat in the realm of bioterrorism and the causative agent *Burkholderia pseudomallei* has been designated a Category B Select Agent by the United States Centers for Disease Control and Prevention. This has stimulated and funded intense research on both the bacterium and the disease of melioidosis. Despite this research, a great deal remains unknown about basic details of melioidosis in regards to the geographic distribution of *B. pseudomallei*, how it is disseminated in the environment, what environmental factors limit its geographic range, and what is the predominant route of natural infection [2-6]. The intricacies of the molecular pathogenesis of host-bacterium interactions, which ultimately determines disease severity and outcome are in need of more research, particularly how the route of infection may affect disease outcome and how host risk factors such as diabetes, chronic lung disease, chronic renal disease, and alcohol abuse influence the pathogenesis of infection [7,8].

Discovering the answers to the many remaining questions about *B. pseudomallei* and melioidosis will require a multipronged approach examining the bacterium at the molecular and ecological levels, animal models of disease, and epidemiological and clinical data from human patients. The scope of this dissertation is focused on the development of novel animal models of melioidosis, particularly aerosol and percutaneous infection in a caprine model system. The vast majority of research to date has been conducted in mice, but new animal models are needed for a

more complete and comparative approach to the study of melioidosis and the evaluation of novel therapeutics.

1.1) Discovery and Early History of Nomenclature

The disease now known as melioidosis was first diagnosed during the postmortem exam of a Burman man in 1911, and subsequently described in the literature as a short report by Whitmore and Krishnaswami in 1912 [1] and then as a series of 38 cases in 1913 [9]. The disease was noted for its similarity to glanders, but was distinguished clinically by the lack of association with equids and by the microbiological characteristics of the bacterium – particularly the rapid growth, wrinkling of colonies on glycerol agar, and motile nature of the bacteria in young culture – which Whitmore coined *Bacillus pseudomallei* [9]. The disease was referred to as Whitmore's disease or morphia injector's septicemia because of its association with morphine addicts (30 of 38 cases had evidence of morphine use) [9,10].

The term 'melioidosis' is derived from Greek roots: *melis*- a distemper of asses, *-oid* similar to, *-osis* a condition, or 'a condition similar to glanders' and was introduced in 1921 by Stanton and Fletcher [10]. They also proposed *Bacterium whitmori* as the scientific name of the causative bacteria. Over time the bacterium has been referred to by many names including *Bacillus whitmori*, *Pfeifferella whitmori*, *Pfeifferella pseudomallei*, *Actinobacillus pseudomallei*, *Flavobacterium pseudomallei*, *Malleomyces pseudomallei* (Breed 1939), *Loefflerella whitmori*, *Loefflerella pseudomallei* (Brindle and Cowan 1951), *Pseudomonas pseudomallei* (Haynes 1957), and finally *Burkholderia pseudomallei* (Yabuuchi *et al.* 1993) [10-12].

1.2) Global Distribution

Melioidosis has classically been viewed as a disease of Southeast Asia and northern Australasia, typically remaining between the latitudes of 20°N and 20°S. While this remains the primary areas of highly endemic and endemic melioidosis – including the subcontinent of India, the range of sporadic autochthonous cases includes Pakistan, Iran, several countries of sub-Saharan Africa, Central and South America, and the Caribbean [13]. Several reports have provided excellent reviews of the known distribution of *B. pseudomallei* and melioidosis, with the remaining caveat that many areas of the world simply lack the diagnostic capabilities to diagnose melioidosis in patients and have not investigated the presence of environmental isolates of *B. pseudomallei* [13-15]. Even within the endemic regions where environmental studies have investigated the presence and distribution of *B. pseudomallei* in soil and water samples, often in relation to seroprevalence data, there is great local and regional variability, which has been seen in studies from Australia [16,17], Papua New Guinea [18], Thailand [19-22], Malaysia [23,24], Lao People's Democratic Republic [25,26], Cambodia [27], Taiwan [28-30], and China [31,32]. This is also believed to be related the level of investigation and diagnostic capabilities, as well as the true variability of the presence of *B. pseudomallei*, which is still poorly understood throughout much of the endemic range of melioidosis [2,33].

1.3) Environmental Microbiology

Examining the spatial distribution of *B. pseudomallei* in relation to landscape, soil, and water properties is a rapidly growing area of melioidosis research. A more complete understanding of the environmental risk factors that are associated with disease as well as the factors that may limit or contribute to the geographic spread of *B. pseudomallei* is needed so that effective prevention and control measures can be taken [2]. The initial association of *B.*

pseudomallei with clay soils at depths of 30 cm [34] is quickly developing into a much more complex picture of the distribution and habits of the bacterium in the environment. In Northern Australia, the presence of *B. pseudomallei* in environmental soil samples has been associated with land use [4]. The presence of animals near sampling sites has been associated with positive soil samples, which was primarily associated with native species (wallabies) [3]. Another study found that positive soil samples were associated with areas containing domestic animals in environmentally manipulated (residential, farming, livestock) areas [4]. In the same study, positive samples from undisturbed areas were typically from areas around creeks (higher water content) and in close proximity to grasses – typically with extensive root systems [4]. This association with grasses has been shown to extend beyond the roots and rhizosphere to the above ground leaves as well, representing a newly discovered niche as well as another means of dispersal through grazing, which could involve both native and domestic species [35]. Additionally, melioidosis in birds, though rare, has also been implicated as a possible mechanism of dispersal for *B. pseudomallei* [36].

Higher water content and season (wet vs. dry) were not as associated in disturbed areas, which could be a result of other compensatory factors promoting persistence and growth of *B. pseudomallei*, such as higher aeration, irrigation/watering, greater nitrogen content, and lower pH [4]. Clay soil alone was not associated with the presence of *B. pseudomallei*, but when associated with oxidized iron (red color) or loam, clay soil was associated with the presence of *B. pseudomallei* [3,4]. An association with sandy soil has also been reported in Thailand, which is believed to allow *B. pseudomallei* to move freely with the flow of water and therefore increase contact with people during the rainy season [37]. In addition to water content >10%, which has repeatedly been shown to be important for detecting *B. pseudomallei* in soil, the authors also

identified higher chemical oxygen demand, total nitrogen, and pH of 5.0 – 6.0 to be significant factors associated with the presence of *B. pseudomallei* [37]. The uneven distribution of *B. pseudomallei* in the environment may be associated with local variations in these physicochemical properties of soil, which has been noted in subsequent studies in Thailand [37,38].

Water bores have also been implicated as a source of infection for *B. pseudomallei* in Northern Australia in both human [17,39] and animal (pig) [40,41] cases and clusters of melioidosis. Surveys of water bores in Northern Australia have shown that 26 to 33% of bores are positive for *B. pseudomallei* [42,43]. The presence of *B. pseudomallei* was associated with soft, acidic water with low salinity and high iron levels [42]. These characteristics seem to favor the persistence of *B. pseudomallei* after initial contamination, but are not essential, as *B. pseudomallei* has been found to survive in triple distilled water for over 16 years [44], highlighting the remarkable ability of the bacterium to survive under inhospitable conditions.

Several papers have examined the range of environmental conditions under which *B. pseudomallei* can grow or survive in a viable but non-culturable state, including ranges of ultraviolet light exposure, pH, salinity, temperature, water content, and on a variety of environmental surfaces [45-49]. A number of review papers have gone a step further to integrate these findings into the broader picture of environmental microbiology, ecology, as well as human and animal interactions, and are an excellent reference on this topic [2,5,50].

One goal of these types of studies is to be able to predict areas that have higher risk for melioidosis based on environmental characteristics. A spatial analysis study conducted in Northern Australia has demonstrated that melioidosis cases can be seen to cluster around certain soil/geological features [51] such that there is the possibility to use geographic information

systems to better understand the ecology of *B. pseudomallei* and determine the temporospatial basis of melioidosis risk more precisely within endemic areas.

1.4) Epidemiology

The epidemiology of melioidosis throughout much of Southeast Asia and Northern Australasia was well summarized by Cheng and Currie in 2005 [14]. The reports for several countries often have been limited to individual case reports, the presence of environmental isolates, or inference – by the importation of melioidosis to non-endemic countries in travelers or animals from endemic regions. Larger prospective and retrospective studies have been repeatedly conducted on the epidemiology and clinical disease from the highly endemic areas of Thailand [52-59], Northern Australia [8,16,60-71], and Malaysia [72-77], but studies examining melioidosis in Cambodia [78-80], Vietnam [81], Singapore [82,83], Taiwan [84-86], India [87], or Brazil [88] in a similar manner have just begun to enter the literature – primarily in the last 5 years. The findings of these studies reflect distinct environmental, socioeconomic, and individual risk factors for melioidosis and mortality from melioidosis in each country/region, as well as differences in clinical disease presentations.

The yearly incidence of melioidosis varies widely across the endemic area, by individual regions, countries, and year to year. These rates are not reported in all studies, but range from a low of 0.19/100,000 in Taiwan [30], 1.7/100,000 in Singapore [75,82], 4.3 to 16.35/100,000 in Malaysia [73,75], 4.4 to 21.3/100,000 in Thailand [52,56,57], and 5.4 to 50.2/100,000 in Northern Australia [8,66]. The high of 50.2/100,000 is the highest reported incidence for melioidosis and was associated with a record 91 cases in a single year of the "big 2009 – 2010 wet season" at the Royal Darwin Hospital [66]. Studies that restrict their inclusion criteria to bacteremic cases must have the reported incidence interpreted with the qualification that they are

under-representing the overall incidence of melioidosis [56]. The 'typical' percentage of melioidosis patients that are bacteremic is 50 to 55% as reported in large epidemiologic studies from Northern Australia [8] and Thailand [57]. Considerably higher incidence of bacteremia has been reported in Taiwan – 75 to 81.1% [84,85], Vietnam - 81.6% [81], and as high as 90.9% in one study in Singapore [82], though a more recent study from Singapore reports bacteremia in 50.4% of melioidosis patients [83]. The higher percentage of bacteremic cases in Taiwan was associated with an outbreak of cases following Typhoon Haitang in July of 2005 [81,84,85]. No such event was documented in the series from Vietnam or Singapore.

1.5) Environmental Risk Factors

Historically, Singapore had been relatively unique amongst endemic countries in that there was no wet season associated increase in melioidosis cases as rainfall is fairly constant throughout the year [82]. However, unusually heavy rain, strong wind, and flash flooding in early 2004 preceded a cluster of melioidosis cases that notably increased the percentage of pneumonic presentations as well as the case-fatality rate [83]. Malaysia also has a weaker, but present, seasonal association with cases based on its more uniform weather pattern [72,89]. The association of cases with the wet/monsoon season is well-established (but often not statistically confirmed as significant) in much of the remaining endemic region, with as many as 81% of cases presenting during the rainy season in Australia [8]. A few studies have shown statistical significance with season [81,90], rainfall [73,87,89], or both [52]. However, the absolute amount of rain does not necessarily correlate with the number of melioidosis cases, as a significant negative correlation with rainfall was reported in one of the largest studies from Thailand [57].

The association with rain has been more closely associated with the intensity and severity of the weather events, particularly monsoons with heavy rain and strong wind [91,92]. The

intensity of rainfall (≥125 mm 14 days prior to presentation) has been significantly correlated as an independently risk factor for pneumonia, bacteremic pneumonia, septic shock, and death in Australia [92]. A more general, but significant, linear association of cases and death with rainfall was also observed in Malaysia [73]. *B. pseudomallei* is believed to be moved towards the soil surface during the wet season as the water table rises [34] and then aerosolized by severe weather events, which result in more pneumonic and severe presentations [92]. However, intense rainfall has been shown to increase the presence of *B. pseudomallei* in groundwater seeps, which could facilitate exposure that is not necessarily associated with inhalation [93].

Soil exposure is another long-established risk factor for melioidosis [52,70]. However, there is a degree of regional variation in the incidence of soil exposure as a risk factor for melioidosis. This is primarily associated with rice farming, with 69.4 to 84.9% of melioidosis patients from Vietnam, Cambodia, and Thailand having occupational exposure to soil through rice farming [52,53,79,81]. Early studies from Northern Australia reported occupational exposure (farmer, stockman, laborer, etc.) in 65% of patients [69]. However, the 20 year prospective study in Darwin, Australia reported only 18% of patients with occupational soil exposure (75% with any soil exposure) [8]. Soil exposure was only a significant risk factor for cutaneous melioidosis, which has a distinct epidemiology from bacteremic or pulmonary melioidosis [64]. A study from India reported 36.8% of patients with soil exposure [87], while another study from Taiwan reported 27% of patients having soil exposure, with only one patient (3.3%) working as a rice farmer [84]. In Singapore, soil exposure is primarily associated with construction workers, which account for 17.3 to 26.9% of melioidosis cases [82,83]. A significant association with occupation (and likely higher levels of soil exposure) was seen in the

industries of farming, fishing, and forestry (combined) and the unemployed, both of which were at higher risk for melioidosis and mortality from disease [73].

1.6) Transmission

The implication of the environmental microbiology and environmental risk factors is that *B. pseudomallei* is typically acquired and results in infection following percutaneous inoculation (soil/water exposure), inhalation (aerosolized bacteria during severe weather), and ingestion (drinking from contaminated water sources). The evidence is strongest for cases with a known preceding injury [8] or clustering around a contaminated water source [17,39], but these represent only a small proportion of melioidosis cases. The importance of each route of infection is supported by epidemiologic data, but not by direct evidence [6]. One murine study has examined the importance of intranasal infection and the development of neurological melioidosis as bacteria directly invade the central nervous system (CNS) through the olfactory epithelium and olfactory nerve [94], which aligns well with the distinction of primary and secondary neurologic melioidosis seen in Australia [8].

Even though *B. pseudomallei* can be isolated from blood, eyes, wounds, pus, sputum, vaginal swabs, urine, and feces [95], transmission between individuals is rare. Transmission between individuals has been reported from close contact, possibly by respiratory secretions [96,97] as well as one case by sexual transmission [98]. The role for sexual transmission has been suggested given the frequency of prostatic infection in Australia, but is challenged by the fact that no cases of sexual transmission have been reported [14,99]. Vertical (transplacental) infection of neonates as well as transmission via breast milk has been documented [100,101]. Neonatal melioidosis is often thought to originate from perinatal transmission, but only 20% of

cases were actually attributed to maternal transmission in a recent review [14,102]. Nosocomial infection has also been reported [102,103] as well as spread from contaminated hand soap [104].

Zoonotic transmission of melioidosis through infected milk from mastitic goats has been a serious concern in endemic regions, as milk from infected goats has cultured positive, but no cases of transmission have been documented [105]. Three cases of suspected zoonotic transmission have been described from Australia [105]. Saprozoonotic infection – animal contamination of the environment (urine, feces, exudates, or carcasses) leading to human exposure and infection – is believed to be a larger concern for human disease [106]. Horizontal spread of *B. pseudomallei* has not been documented in animals apart from nosocomial infection [105], but vertical transmission has been documented in a goat and pig [105,107].

1.7) Individual Risk Factors

While these environmental aspects are important risk factors for melioidosis, the greatest and most consistent risk factors are associated with predisposing conditions in individual patients. These host-associated factors are also believed to play the most important, but poorly defined, role in the highly variable nature of disease presentation, progression, and outcome [63]. Male gender predominates, 58.5 to 88% of patients, in all melioidosis case series (excluding pediatric and cutaneous studies) [57,83]. This is presumed to be associated with increased environmental exposure, similar to the higher incidence of melioidosis in Australian Aborigines [8,62]. However, the statistical significance of male gender has only been reported in a limited number of studies [16,57,62,73,83]. Older age is also a well-established risk factor for melioidosis, with the typical mean/median patient age ranging from 42 to 65 years; age ≥ 45 is a statistically significant risk factor for disease [57,62,83], and age ≥ 50 is significantly associated with death from disease [8].

1.7a) Diabetes

The most common comorbidity associated with melioidosis is diabetes mellitus, which is present in approximately 50% (range 25 to 75.8%) of melioidosis patients, either as known diabetics or newly diagnosed diabetics at presentation [81,87]. Nearly all diabetic patients are type 2, adult onset diabetics [8]. The relative risk for melioidosis in known diabetics is 12.4 compared to 7.8 for undiagnosed diabetics [57]. Several studies have demonstrated a statistically significant association between diabetes and melioidosis [8,16,53,59,62,65,73,82,83]. One study established diabetes as a significant risk factor for bacteremic melioidosis [53], but only two studies have shown it to be independently associated with mortality [73,82].

The presumptive mechanisms for increased susceptibility in diabetics are related to defects in innate immunity, particularly neutrophil function, which is essential in the response to infection with *B. pseudomallei* [108]. Several immune defects seen in diabetes have been reported that are likely of importance in melioidosis. Neutrophil adhesion has been found to be reduced [109,110] or increased [111,112] in diabetes. While these finding have been inconsistent, possibly because of different methodologies, the authors suggest that neutrophils are unable to effectively exit the vasculature. This defect is compounded by decreased neutrophil chemotaxis [111,113,114], limiting the ability of neutrophils to reach the site of infection. Bacterial phagocytosis [115-117] and killing [115,116,118-123] are reduced as well. Oxidative burst generation, which is closely associated with killing, is slightly more complex since diabetes is a disease associated with high oxidative stress. Basal free radical production in diabetic neutrophils is higher than in non-diabetics – suggesting spontaneous activation, but after stimulation, free radical production was less than healthy controls [111]. Better glycemic control

in type 2 diabetics also has been shown to increase the respiratory burst production as well as the production of superoxide in stimulated neutrophils [124].

Monocyte functions, including phagocytosis and respiratory bust activity, have also been found to be impaired in diabetics [125-127]. Multiple abnormalities of cytokine production have been reported in type 1 and type 2 diabetics [128-130]. Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 are also increased in obesity [131] (a typical comorbidity of type 2 diabetes) and have been implicated in cytokine abnormalities seen in type 2 diabetes [132]. Most notably, diabetic patients have been shown to have strongly reduced IFNγ production [133,134]. The apparent theme of the diabetic immune system is that the basal state is one of chronic activation, but when it encounters a pathogen, the response is suboptimal [130]. Many of the aforementioned immune dysfunctions appear to parallel several mechanisms of immune subversion by *B. pseudomallei* (discussed in Sections 1.9 and 1.10), suggesting that the increased risk of melioidosis in diabetics could be the result of additive immune dysfunction.

Limited *in vitro* studies of immune function in response to *B. pseudomallei* have been performed using diabetic leukocytes. Diabetic human polymorphonuclear neutrophils showed significantly decreased phagocytosis, increased intracellular survival, and reduced post-activation migration in response to *B. pseudomallei* [135]. Interleukin (IL) -17 expression has also been shown to be reduced in *B. pseudomallei* infected peripheral blood mononuclear cells in diabetic patients [136]. This was suggested to be related to defects in T-cell function, but no further studies have been performed to determine the importance of this finding [136]. A recent whole-blood assay identified several differences in oxidative burst activity, pathogen recognition receptor expression on polymorphonuclear cells and monocytes, and inflammatory cytokine expression in response to *B. pseudomallei* in blood samples from poorly controlled type 2

diabetics, well controlled type 2 diabetics, and non-diabetic patients [137]. The overall findings suggested that type 2 diabetics have defects in leukocyte activation and generate an exaggerated inflammatory response in melioidosis [137].

Murine models have more consistently show defects in macrophage function as well as variable defects in dendritic cell function (associated with the duration of hyperglycemia – acute vs. chronic) [138,139]. Delay (~24 h) in the host innate immune response to *B. pseudomallei* was seen using microarray transcriptional analysis in streptozotocin-induced diabetic mice compared to normoglycemic controls, which may account for the increased susceptibility of diabetics to melioidosis [140].

1.7b) Excessive Alcohol Consumption

Hazardous alcohol use was present in 39% of Australian melioidosis patients, making it equally common as a risk factor as diabetes in the study [8]. The mechanism of susceptibility is again believed to be related to dysfunction of innate immunity and polymorphonuclear leukocytes [60], which has been shown in injury and sepsis models of binge drinking [141-143]. It is believed that the importance of hazardous alcohol use is from the acute defects in neutrophil function and innate pulmonary immunity caused by high blood alcohol content, rather than chronic liver disease [8]. Excessive alcohol consumption also has been reported in other endemic regions, Taiwan – 20%, Thailand – 12%, Cambodia – 12%, India – 10.5% and Vietnam – 5%, though it was not a statistically significant risk factor in any of these studies [53,80,81,84,87]. The prohibition of alcohol consumption, essentially eliminates it as a risk factor in Muslim countries such as Malaysia [74].

1.7c) Renal Disease

A similar underlying pathologic mechanism of neutrophil dysfunction has been proposed for the association of chronic renal disease and melioidosis [8,60,62]. Renal disease was reported as a statistically significant risk factor for melioidosis in two studies from Thailand [53,59], bacteremic melioidosis in India [87], and further statistically associated with mortality in studies from Taiwan [84], Singapore [82], and Australia [62]. However, a subsequent follow-up to the Australian study using a larger data set confirmed renal disease as a risk factor for disease, but not mortality from melioidosis [8]. The reported incidence of renal disease in Thai melioidosis patients, 20 to 27% [53,59], is higher than reported for Australia, which ranges from 7 to 12% [8,60,62,63,66]. This may be, in part, from the higher frequency of renal tubular acidosis and calculi formation seen in the Thai population [144], which may result in a higher incidence of chronic renal disease/failure. One report from Malaysia also mentioned calculi in the description of renal impairment [76], though most other studies restrict the definition of renal disease to chronic elevations in serum creatinine [62,81,84], and no report has specifically identified calculi alone as a risk factor for melioidosis.

1.7d) Other Risk Factors

Chronic lung disease has been shown to be significantly associated with melioidosis risk, specifically for pulmonary melioidosis in Australia, being present in 26% of patients [8,65]. The underlying mechanism of increased susceptibility was hypothesized to relate to impaired alveolar macrophage function [8,65]. Three percent of Malay and nine percent of Indian melioidosis patients were reported to have chronic lung disease [74,87]. Thirty-three percent of Cambodian patients diagnosed with acute lower respiratory infections and melioidosis were also found to have pre-existing chronic lung disease [79]; however, there was not statistical significance to this

finding in any study from Malaysia, India, or Cambodia. Thalassemic disease has also been reported as a significant risk factor, found in 7.3% of melioidosis patients in Thailand [53]. Cardiac failure was also significantly associated with death from melioidosis in one study [82], but no other study has reported it as a significant risk factor for disease or death.

Other diseases or concurrent conditions have been associated with melioidosis, but have not been shown to be statistically significant risk factors. These include cardiovascular disease (rheumatic heart disease, hypertension, stroke), malignancy, kava use (Australia), malaria, trauma, tuberculosis, near drowning, autoimmune disease, and immunosuppressive therapy (typically corticosteroids) [8,52,63,74,84]. Additionally, splenectomy, aplastic anemia, chronic granulomatous disease, dengue hemorrhagic fever, renal transplantation, systemic lupus erythematosus, glucose-6-phosphatasae deficiency, hemosiderosis, cystic fibrosis, and porphyria cutanea tarda, have been reported as potential clinical risk factors in case reports [14]. Although human immunodeficiency virus (HIV) infection has been reported in some studies as a risk factor/predisposing condition, sometimes under the larger heading of immunosuppression [8,74,79,87], two studies have demonstrated that unlike in the case of tuberculosis, HIV infection does not influence the incidence, presentation, or outcome of melioidosis [145,146]. The implications of this finding will be discussed in Section 1.10 on the host immune response to melioidosis.

No concurrent condition or risk factor for melioidosis was identified in 13 to 36% of patients, though the degree to which concurrent diagnoses were pursued was variable depending on the study, particularly in studies with high acute mortality [8,53,62,76]. Patients without risk factors who receive prompt appropriate care are considered to be at very low risk of mortality from melioidosis, while the presence of at least one risk factor is a significant independent

predictor of mortality from melioidosis [8,83]. Interactions between risk factors have also been infrequently reported, specifically between aboriginal ethnicity and diabetes, with an increased relative risk of 20.6 for melioidosis [62]; occupational exposure and diabetes [53]; and an interaction between age and diabetes, with a significant influence of older age on the presence of diabetes [73].

1.8) Clinical Presentations of Melioidosis

The vast majority (85%) of melioidosis cases tend to be acute presentations (symptoms present for less than two months), while 11% of presentations are chronic, and 4% appeared to be reactivation of latent disease [8]. Patients with chronic disease were significantly less likely to be diabetic, have any identified risk factor for melioidosis, or die from disease [8]. An event associated with infection is generally only recognized in a quarter of cases. In those cases, the incubation period ranged from one to 21 days with a mean of nine days [61]. However, cases of latent infection for years to several decades have been documented [147-149]. The temporal distribution of cases predominating in or just after the wet season supports the idea that most cases are truly acute in nature.

In the mid 1980's, the mortality rate associated with untreated melioidosis was approximately 90% without treatment and 80% with treatment. The greatest reduction in mortality from melioidosis was associated with the advent of ceftazidime therapy, which essentially halved the mortality rates associated with severe melioidosis beginning in 1988 [150]. Mortality from melioidosis varies greatly between studies. Currently, this is primarily dictated by access to appropriate medical therapy, which is largely determined by the economics of the various countries within the endemic region. Limited access to treatment often results in later presentation and therefore more severe disease at presentation, which can be compounded by a

lack of appropriate therapeutics, especially in rural areas. This is evident in the current mortality rates for Thailand, Malaysia, Vietnam, and Cambodia , which remains between 40 to 55% [57,75,80,81], while Australia and Singapore have mortality rates of 14% and 16.2% respectively [8,83]. The importance of availability and cost of therapeutics was specifically highlighted in one study from Cambodia where 23 of 24 patients that had fatal disease had no antibiotic therapy, ceftazidime was not available for any patient, and over 60% of patients had to incur debt or sell property (land/livestock) to pay for illness-related costs [79].

Regardless of geographic location, the majority of deaths from melioidosis tend to occur early, usually within two to four days after presentation [8,59,72]. The preponderance of deaths are associated with septic shock [8], which has been significantly associated with death from melioidosis [80,84,87]. Death from septic shock in melioidosis has further been shown to be significantly greater that death from other bacterial infections causing septic shock, though not greater than death from *Pseudomonas spp.* causing septic shock [59]. Bacteremia alone has been associated with melioidosis mortality in several studies [53,55,73,80,82,83,87]. Respiratory failure [84] and inappropriate antibiotic therapy [72,80] have also been found to be significantly associated with mortality in melioidosis patients.

Given that melioidosis is known as a "remarkable imitator" [151], it is generally well-accepted that there is no typical presentation of disease. However, there are patterns of disease and manifestations of melioidosis that are more common than others. Some of these appear to relate to incompletely understood regional factors. Melioidosis is the most common cause of community acquired bacteremia in Northeast Thailand [52] and the most common cause of fatal community-acquired septicemic pneumonia in Northern Australia [60]. Acute suppurative parotitis appears to be a syndrome of melioidosis that is primarily restricted to pediatric patients

in Southeast Asia (Thailand, Cambodia, Malaysia, and India) [152-155] and has only been reported once in Australia from the Torres Strait islands [156]. Hepatic and splenic abscesses are also much more frequently found in Thai patients than their Australian counterparts [58], while prostatic abscesses and CNS melioidosis tends to be more common in Australia and are more rarely identified in Thailand [8].

Primary cutaneous melioidosis is a typically less severe but relatively common form of disease that has a distinct set of risk factors and clinical presentation, which does not appear to relate to any geographical distribution [64]. Musculoskeletal (septic arthritis, osteomyelitis, pyomyositis, or soft tissue) infections of melioidosis are also well recognized manifestations of disease that are occasionally primary but more often secondary in nature [157-160]. Numerous additional sites of infection have been described as well, including pancreatic [161], renal [162], adrenal [163], breast (mastitis) [101,164], scrotal [165], and neck abscesses [166], mediastinal lymphadenopathy [167], mycotic aneurysm [168], pericarditis [169], sinusitis, acute and chronic otitis media [170,171], and corneal ulcers [172].

1.8a) Pulmonary Melioidosis

Pneumonia is typically recognized as the most common presentation of melioidosis, being present in approximately 50% of patients, though the reported incidence varies from 20 to 87% [14,59,85]. One study reports the development of secondary pneumonia in 20% of patients, often following a documented bacteremia from a previously identified primary site of infection [65]. The designation of primary pneumonia does not necessarily indicate evidence of inhalational infection since lung involvement after a known inoculation event is well documented [14]. However, inhalational infection is increasingly recognized as an important route of infection based on epidemiologic data demonstrating high rates of pneumonic

presentations after severe weather events and low risk for contact with contaminated soil or water [83,85,91,92].

Several studies have specifically examined pulmonary melioidosis in prospective and retrospective case series as well as review articles [65,79,173-175]. However, only one has identified specific significant risk factors for primary melioidosis pneumonia compared to other presentations of melioidosis, which include, rheumatic heart disease/congestive heart failure, chronic obstructive pulmonary disease, smoking, and diabetes [65]. Pneumonic presentations are also independently significantly associated with higher rates of septic shock and death [65,74].

The presenting clinical signs, laboratory, and radiographic findings of pulmonary melioidosis are quite variable and are often dependent on the nature of disease – acute, subacute, or chronic [174]. Clinical signs and clinical pathological findings are non-specific and often include fever, cough, dyspnea, thoracic pain, hemoptysis, and leukocytosis [79,173,175]. Sputum culture can be diagnostic as it is 100% specific, though it is negative in 32% of patients with radiographic evidence of pulmonary involvement. Positive sputum culture is a significant independent predictor of mortality from melioidosis [176].

Thoracic radiographic findings are also highly variable – within and between studies – and there are no pathognomonic Roentgen signs for melioidosis [177]. Radiographic signs in acutely presenting cases often have local, patchy, alveolar infiltrates, or disseminated nodular lesions, which are suggestive of metastatic (hematogenous) spread [175,178-181]. Acute/subacute cases have significantly more lower lobe and multilobar involvement, the latter of which is significantly associated with increased mortality when compared to unilobar disease [65]. The findings in subacute to chronic presentations are more variable, with some studies reporting the absence of a predominant lesion [178], while others report findings very similar to

what is seen in acute cases [175]. The upper lobes appear to be more affected in melioidosis, especially in subacute/chronic cases [65,174,175,178,180-183]. Another common finding in humans is cavitary lesions, which have been reported in acute cases, but tend to be more common in subacute/chronic cases [174,175,178,180-182].

1.8b) Septicemic Melioidosis

Bacteremia is typically detected in half of melioidosis patients, though the overall picture of disease among bacteremic patients can be highly variable based on the primary presentation [6]. Patients without a primary site or source of infection other than the blood usually represent 9.5 to 24% of cases [8,74,76,84,87], but one early study found no source of infection for just over half of the patients [59]. The failure to detect a primary site of infection may relate to the diagnostic capabilities and/or the time available to diagnose a primary source prior to death, which is often acute in melioidosis patients, particularly those in septic shock. Both of these may be relevant factors in many parts of Southeast Asia where primary septicemias are documented more frequently [63]. However, true differences in bacterial, environmental, or host factors could also account for this difference in presentation. The development of secondary foci is common in these patients, with 42% of patients showing later infections in the lungs, spleen, joints, prostate, liver, bones, CNS, or kidney [8]. The lack of a primary site of infection was significantly associated with mortality in one study [74], but the overall significance of this finding is less clear since it has not been found in other studies. Patients with repeated positive blood cultures [55] as well as higher absolute number of CFU/mL at presentation were found to have significantly higher mortality (regardless of primary site of infection, if any) [184]. Patients having >100 CFU/mL showed a 96% mortality rate and patients with ≤ 1 CFU had a mortality rate of 42% [184]. However, there was no difference in mortality between melioidosis and other

gram negative associated bacteremia when the number of CFU/mL was <10, which accounts for more than 75% of bacteremic melioidosis patients [184,185]. Only with bacteremia >10 or >50 CFU/mL was *B. pseudomallei* bacteremia significantly associated with mortality compared to other gram negative bacteria, which the authors acknowledged could have been biased by the selection of more severely ill patients for use in the study [184].

1.8c) Genitourinary and Visceral Melioidosis

The involvement of the genitourinary system overall in melioidosis is generally reported around 10 to 15% [14,59,63,84]. However, prostatic abscesses dominate the reports of genitourinary melioidosis, largely because of their high frequency in male Australian patients and their rarity in other endemic regions [14]. Independent risk factors for prostatic abscess are hazardous alcohol and kava use. Prostatic involvement was present in 21% of male melioidosis patients, being the primary presentation for 96% of these patients [186]. Notably diabetes was not associated with prostatic disease, and another Australian study reported that no patient with primary genitourinary disease had diabetes [63]. However, significantly more patients with prostatic abscesses were bacteremic than patients without prostatic involvement [186]. Ninety-three percent of patients had at least one urinary symptom, an abnormal prostate exam, abnormal urinalysis, or positive urine culture; while, nearly all (99%) of patients could be diagnosed by CT exam [186]. The high clinical awareness of prostatic disease and its association with melioidosis in Australia has resulted in intensive diagnostics in these cases, which could explain some the higher levels of reported prostatic disease associated with melioidosis in Australia as compared to other countries. Given the relative sensitivity of urine culture (89%) to detect prostatic disease (Australian study), the fact that only 23% of Thai patients had a positive urine cultures and only 5% had a positive culture and signs of urinary tract infection, it seems likely that there is a true

difference in the incidence of melioidosis associated prostatic disease between Australia and Thailand [144,186]. However, increasing awareness and diagnostics in Thailand have resulted in the detection of more cases of prostatic melioidosis (0.4 to 2.5% of visceral abscesses) and further found that *B. pseudomallei* is the causative agent of 50% of prostatic abscesses in Thailand [58,187,188].

Involvement of the female reproductive tract is rarely reported in melioidosis studies [99]. Most reports relate the involvement of melioidosis of the female reproductive tract to pregnancy and the development neonatal melioidosis via vertical transmission [100,102].

Renal abscesses are the next most reported type of genitourinary involvement, which appears linked with renal calculi in Thailand [144], but is commonly seen in patients with prostatic abscesses in Australia [186]. Early retrospective studies from Thailand reported renal abscessation in 12% of patients [162,188]. This number appears to be highly skewed by the retrospective selection criterion of having an abdominal ultrasound performed, as a recent prospective study only reported renal abscesses in 0.9% of melioidosis patients [58], which is closer to the reported incidence of 3% from a prospective study in Australia [8]. Regardless of the source of bacteria, positive urine culture has been shown to be an independent predictor of mortality in Thai patients [144].

Splenic and liver involvement in the form of solitary, but more often multiple, abscesses are common in melioidosis [6]. Abdominal pain is significantly associated with the presence of one or more intra-abdominal abscesses in melioidosis, though 27% of cases were clinically silent in one prospective study [58]. An earlier retrospective study reported that two-thirds of patients reported abdominal pain or tenderness [188]. The only identified significant risk factors for intra-abdominal abscess were younger age (median 48, vs. 54 years) and known pre-existing

renal disease [58]. Lower mortality in the group with abscesses approached but did not achieve statistical significance. This failure to achieve significance may have been affected by the fact that the study population was skewed such that patients that died within the first 48 hours (typically representing half of all deaths), were under-represented. It is hypothesized that the formation of abscesses in an indication of more successful host immune defenses and isolation and control of infection [58].

The incidence of splenic and hepatic abscesses is notably different in Thailand and Northern Australia. Diagnosis is often achieved by ultrasound, but as in the case of pulmonary and prostatic melioidosis, CT imaging is more frequently used and has certain diagnostic advantages [189]. Early retrospective studies initially reported very high incidences of splenic (74%) and hepatic (46%) abscesses in Thailand [162,188]. As in the case of renal abscesses, much lower incidences of spleen and/or liver abscesses (33%) were reported from the recent prospective Thai study [58]. The prospective findings were very similar to the previously reported incidence of 32% for splenic and/or hepatic abscesses in Thai pulmonary melioidosis patients [175]. While this incidence is much lower than previously reported, it remains much higher than the Australian incidence of 5% and 3% for splenic and hepatic abscesses, respectively [8]. The reason for this regional difference is unclear, but again may relate to strain variation or duration of illness. Access to intensive care is often limited in certain geographical regions of Thailand, which may prevent prompt treatment, resulting in more advanced disease at the time of presentation [6,8,57].

1.8d) Central Nervous System Melioidosis

Central nervous system involvement is rare, but has been documented in both human and natural and experimental animal infection [190]. It occurs in 4% of Australian cases and 0.2 to

1.5% of Thai cases [8,191,192]. The seriousness of neurological melioidosis has made it a highly studied disease relative to its incidence and was well reviewed by Koszyca *et al.* in 2004 [193]. The higher incidence of neurologic melioidosis in Australia compared to Thailand is well-recognized, as is the differences in the specific features of neurologic disease in each location. In Australian cases, brain stem encephalitis and meningoencephalitis tend to predominate [190,193,194] with infrequent reports of cerebral or other CNS abscesses [8]. The findings in Thailand [191,195,196], Singapore [197], and Malaysia [198] are just the opposite, with the predominant form of CNS melioidosis cases presenting with cerebral abscess. The exception to this is in pediatric neurologic melioidosis where cases often lacked an evident focus or presented with meningitis [199].

Primary CNS melioidosis is believed to often occur through direct invasion of the brain through olfactory neurons, which has been demonstrated experimentally in mice [94]. This is indirectly supported by a significant difference in bacteremia observed in primary and secondary neurological melioidosis patients, with all of the secondary cases being blood culture positive and all of the primary cases being blood culture negative [8]. Experimental infection in mice has shown that *B. pseudomallei* can readily cross the blood-brain barrier following enteral infection [200]. It was also noted that four of the five cased in the study from Singapore were concurrently diagnosed with sinusitis at the time of CT examination, suggesting involvement of the olfactory epithelium in the entry of *B. pseudomallei*, but no sinus cultures were performed [197].

1.8e) Cutaneous Melioidosis

Skin or soft tissue involvement is relatively frequently reported in melioidosis studies, 29% in Cambodia [80], 24% in Malaysia [76], 17.5% in Singapore [82], 16% in Thailand [59], 5

to 13% in Australia [8,63,66], and 3.3% in Taiwan [84]. Only one study has prospectively examined cases of cutaneous melioidosis and subdivided cases into primary and secondary cutaneous lesions, which were present in 12% and 2%, respectively, of all melioidosis patients [64]. This study revealed several unique findings particular to cutaneous melioidosis. Typical risk factors of diabetes, hazardous alcohol consumption, and chronic lung disease were all significantly less common in patients with cutaneous melioidosis. Patients were significantly more likely to be children ≤15 years of age and have occupational soil exposure. Primary cutaneous melioidosis patients more often presented with chronic disease (≥ 2 months duration), had lower incidences of bacteremia (1.7%) or dissemination (7%), and did not develop severe sepsis or die [64].

1.8f) Pediatric Melioidosis

Cases of pediatric melioidosis are reported from most endemic regions, where they typically account for a small percentage of overall cases. The highest report of 16.7% of melioidosis cases occurring in children was reported in Thailand in 1989, but a later and larger study reported that only 9% of cases were children [57,152]. Cases of pediatric melioidosis accounted for 5.5 to 8.4% of melioidosis in Malaysian studies [73,201], while Australian reports indicated 5 to 5.3% of cases were children [8,202,203]. The lowest occurrence of pediatric melioidosis is reported in Singapore where only 2.4% of cases were in children less than 15 years of age [82]. Some caution must be used when comparing studies, as many applied different age divisions to define pediatric cases.

The findings in these pediatric series are often quite different than adult or whole population studies; however, drawing conclusions from the pediatric studies is often difficult because of the low number of cases. In general, the majority of pediatric cases present during

the wet season, though this was not seen in studies from Cambodia and Malaysia [78,201]. The male skewed gender distribution is less pronounced in pediatric cases, with some studies showing a majority of males [153,203-207], while other report fairly even numbers [152,202], and one study reporting more girls affected than boys [78], and none of the differences were statistically significant. Predisposing risk factors for melioidosis were also less common in several pediatric studies, with no cases [152,153] or <10% of cases [78,205] having any known or potential risk factors. Other studies reported risk factors in 25 to 68.8% of children diagnosed with melioidosis [201-204,206,207]. A prior episode of dengue hemorrhagic fever was identified as a potential risk factor in children for disease from melioidosis [206,207], but neither this association nor any other predisposing condition was significantly associated with melioidosis in children. The lack of any risk factors in children with acute suppurative parotitis was significantly different than the presence of risk factors in adults with melioidosis [152]. Pediatric patients also differed significantly between systemic and local disease groups, with the presence of underlying disease, septic shock, and death all being less in patients with localized disease [206].

However, the greatest difference in pediatric and adult melioidosis is in the clinical presentation of disease, some of which may relate to the frequent absence of predisposing risk factors. In Thailand, Cambodia, and Malaysia localized disease accounted for more than half of all pediatric patients. While the absolute number of deaths were comparatively uncommon relative to adults, death was typically associated with disseminated disease and septic shock within 48 hours of presentation, which had mortality rates ranging from 60 to 100% [78,153,201,206,207]. Local disease only produced one case fatality and often responded very well to surgical drainage and what would otherwise be considered inadequate antibiotic therapy

[201,205]. The most common forms of local disease are soft tissue infections of the head and neck, which accounts for approximately 80% of local disease [78,153]. Acute suppurative parotitis is the most common and well-recognized form of this type of infection, accounting for 33 to 56% of all localized melioidosis cases in children [78,152,153,155,201,205,206].

In Australia, acute suppurative parotitis is virtually non-existent in children or otherwise, with only one case reported [61]. Pediatric disease is generally rare in Australia when compared to much of Southeast Asia, but has similar presentations of disease compared to the adult population in Australia. The main differences are that underlying diseases are much less common in pediatric cases and mortality is lower [203]. Additionally, within the pediatric cases, neurologic disease is over represented and significantly more common in children than adults, often resulting in death or permanent neurological deficits [202,204]. The features of pediatric neurologic melioidosis cases in Australia have been similar to those seen in adults, with brainstem involvement predominating [202,204]. Central nervous system melioidosis has been reported less frequently in children in Thailand, particularly as a primary presentation, and again is similar to adult cases that often present with cerebral abscesses [196].

1.8g) Regional differences in the clinical presentation of melioidosis

There are several well-recognized clinical differences in Australian and Thai melioidosis patients, which have been mentioned in the preceding sections. The high incidence of genitourinary (primarily prostatic abscesses), virtual absence of suppurative parotitis in children, and encephalomyelitis are all features of disease in tropical Australia that are not seen in Thailand [14]. A possible explanation for these differences is a regional variation in the *BimA* gene of the bacterium, which has been shown to be different in Australian and Thai isolates [208]. Differences in human leukocyte antigen profiles have also been found in certain subsets

of melioidosis patients [209], which could also potentially influence regional differences. The occurrence of suppurative parotitis in Thai children has also been suggested to be related to behavioral and/or dietary factors – bathing or swimming in, and drinking untreated water – resulting in higher levels of exposure and therefore disease [78,203].

Seroprevalence data strongly support higher levels of exposure in Thai children, with 60 to 80% of children having a demonstrable indirect hemagglutination assay (IHA) titer by age four [54,210,211], whereas only five to seven percent of Australians in north Queensland are seropositive [212]. Furthermore, IHA titers of 1:40 are considered reactive in Australia, while a titer must be ≥1:160 to be considered reactive in Thailand [211,212]. It has been suggested that cross-reactivity with antibodies to *B. thailandensis* may account for the higher and move prevalent titers to *B. pseudomallei* in Thailand, as *B. thailandensis* is not present in Australia and there is a similar incidence of melioidosis in both countries [54]. Studies examining this cross-reactivity in the IHA have had mixed results [212,213]. While one study specifically used the cross-reactivity of antibodies to *B. pseudomallei* and *B. thailandensis* to aid in the development of an immunofluorescent antibody test that is compatible with basic microbiology facilities and safe for laboratory personnel [214]. The importance of this cross-reactivity and/or high seroprevalence for *B. pseudomallei* will be further discussed in Section 1.11 on diagnostic testing.

1.9) Pathogenesis

The molecular pathogenesis of infection of *B. pseudomallei* is an area of intense research. While a great deal has been learned about the structural and functional aspects of bacterial components, their regulation, and role as virulence factors many aspects of pathogenesis are incompletely or poorly understood. A detailed review of the molecular and cellular pathogenesis

of melioidosis is beyond the scope of this literature review, which will briefly describe the putative virulence mechanisms of *B. pseudomallei*. However, several papers and book chapters are available that provide an excellent resource on this topic [7,10,215-219].

B. pseudomallei is a fairly typical member of the *Burkholderia* genus, which is in the Class Betaproteobacteria. It is gram-negative bacillus that exhibits bipolar staining ('safety pin' appearance) as a result of poly-β-hydroxybutyrate accumulation [219]. Cells are approximately 0.8 x 1.5 μm, and have two to four polar flagella that confer motility that is readily observable in liquid media [10]. Colony morphology is typically dry and rough on selective media, but great variation in colony morphology is seen based on the media used as well as inter- and even intra-strain variability such that pure cultures can grossly appear with mixed colony types. The variation in colony type has been further associated with adaptation to adverse conditions and the expression of virulence factors [220-222].

The capsule of *B. pseudomallei* (type I O-antigenic polysaccharide) is generally viewed as an important virulence factor since infection studies with acapsular mutants show greatly reduced virulence [223-226]. The mechanism responsible for the virulence of the capsule is less clear. The capsule has been shown to reduce the deposition of complement factor C3b, blunting the complement cascade and decreasing bacterial phagocytosis [227]. However, an *in vitro* study using a capsular mutant demonstrated that the capsule inhibits epithelial cell invasion (greater invasion with acapsular mutant), but did not affect intracellular multiplication, increase cytotoxicity, or apoptosis [228]. Decreased virulence associated with acapsular mutants may be the result of a weaker Th1 response, which was observed in mice with equal organ burdens of wild type and capsular mutants, indicating the importance of the capsule in determining the host response and outcome [225].

While the capsule does not appear to be directly involved in adhesion, Type 4 pili, encoded by the *pilA* gene, are important for the adhesion of *B. pseudomallei* to certain types of eukaryotic cells [229]. The *boaA* and *boaB* genes have also been found to encode for respiratory tract adhesins in the autotransporter family [230]. Flagella have a less clear role in the virulence of *B. pseudomallei* in regards to adherence and invasion, with flagellar mutants showing variable *in vivo* virulence depending on species and route of infection, and variable *in vitro* adhesion and invasion of mammalian cells [231,232].

Lipopolysaccharide (LPS) or type II O-antigenic polysaccharide has also been demonstrated to be a virulence factor, with attenuation of virulence in mice, hamsters, guinea pigs, and infant diabetic rats infected with LPS mutants, which is partially due to reduced killing of bacteria by the alternate complement pathway [226,233]. While LPS from *B. pseudomallei* is a virulence factor, it is notably different than classical enterobacterial LPS and is different from the closely related LPS of *B. thailandensis*, with *B. pseudomallei* LPS demonstrating weaker pyrogenic and immunogenic activity [234,235]. One study indicated that signaling for *B. pseudomallei* LPS was through toll-like receptor (TLR) 2, which was primarily responsible for modulating the *in vivo* immune response, as opposed to TLR4, which is the typical receptor for LPS [236]. A subsequent study did not support this finding and supported TLR4 as the primary receptor for *B. pseudomallei* LPS, but relied on *in vitro* findings with killed bacteria [237]. Further evidence of the importance of TLR2 signaling in melioidosis came from the finding that TLR2 expression is upregulated in acute murine melioidosis [238]. Type III and type IV O-antigenic polysaccharide mutants have also shown reduced virulence in mice, supporting their role as virulence factors, though their mechanisms of action are still unknown [224].

The type three secretion systems (T3SS) – functionally a molecular syringe that can deliver effector proteins through eukaryotic cell membranes – are associated with several aspects of virulence in *B. pseudomallei*. Three T3SS are present in *B. pseudomallei*, with T3SS3, being most strongly associated with virulence [239]. The components of the T3SS include: structural proteins BsaQ, BsaU, and BsaZ; translocator proteins BipB and BipD; and effector proteins, BopA, BopB, BopC, and BapE [7,218]. Subsets of different T3SS structural, translocator, and some effector proteins have been shown to be required *in vitro* for optimal invasion of non-phagocytic cells, escape from endocytic vesicles, multinucleate giant cell (MNGC) formation, and stimulation of apoptosis, with mutants typically showing attenuated invasion, delayed endocytic escape, reduced MNGCs and cell-to-cell spreading, and delayed apoptosis [7,218]. The attenuation of virulence *in vivo* for these mutants has been demonstrated except for the effector protein mutants [7]. This is believed to be related to the fact that effectors are typically delivered in concert and likely have additive effects such that the deletion of a single effector will likely have little effect on overall virulence, while mutations in the structural and translocator genes will inherently have wider effects [215]. A more detailed review of the T3SS in *B. pseudomallei* was published by Sun and Gan in 2010 [240].

Actin-based motility is another virulence mechanism of *B. pseudomallei* which allows for the cell-to-cell spread of bacteria. Actin-based motility is well described in several genera of bacteria [241], but the mechanism in *B. pseudomallei* is unique and appears to be essentially dependent on the autosecreted protein BimA [242]. The most common mechanism of actin-based motility uses the Arp2/3 complex, which is present in *B. pseudomallei* but is not essential for actin based motility [243]. Mutations of *bimA* alone will abolish actin-based motility in *B. pseudomallei*, preventing the formation of membrane protrusions and cell-to-cell spread, but it

does not affect endocytic escape [242]. Attenuation of *in vivo* virulence for BimA has only been reported as unpublished data in murine melioidosis [244].

Six type six secretion systems (T6SS) have been identified in *B. pseudomallei* [245], though their roles in virulence remain poorly defined. Mutation of tssH (from T6SS-1) has demonstrated mild reductions in actin-based motility in *B. pseudomallei*. Much more significant reductions in the formation of MNGCs, intracellular growth, and cytotoxicity were seen *in vitro*, as well as a significant reduction of *in vivo* virulence, associated with this mutation [246]. The formation of MNGCs is believed to be important for the local dissemination and immune evasion of *B. pseudomallei*, which is also partially dependent on functional BipB and RpoS [218].

Quorum sensing is a system of density-dependent cell-to-cell communication that is found in many Gram negative bacteria. In *B. pseudomallei*, N-acyl-homoserine lactones (AHLs) are relatively well described, though the complex communication network involved in their signaling and effects is still incompletely understood [247]. Additionally, 4-hydroxy-3-methyl-alkylquinolones have also been described as a quorum sensing system in *B. pseudomallei*, but its functional role has not been determined [248,249]. Quorum sensing affects the regulation of several bacterial factors, many of which are putative virulence factors as well. These include metalloproteases, siderophores, phospholipase C, and biofilm formation [217,250]. Synthesis of AHLs is accomplished by LuxI proteins (three homologues) and regulated by LuxR transcriptional regulators (five homologues). Disruption of genes encoding for these proteins has been shown to decrease *in vivo* virulence, but not exoproduct (lipases, proteases) production or secretion [251-253]. *In vitro* and *in vivo* attenuation of *B. pseudomallei* has also been demonstrated by disruption of signaling through small modified nucleotides

(hyperphosphorylated guanosine molecules), which have been associated with stress, virulence, and survival responses in bacteria [254].

B. pseudomallei produces many active molecules and many of these are secreted though the type two secretion system (general secretory pathway), including proteases, lipases, lecithinases, phospholipases, metalloproteases, serine protease, tyrosine phosphatase, siderophores, and hemolysins [7]. While many of these molecules have been suggested as putative virulence factors based on *in vitro* activity, there is not a correlation with *in vivo* virulence [10,255]. Studies examining the effect of knockout of specific molecules or even the entire general secretory pathway have not shown any decrease in virulence [256,257].

A final group of factors that are not virulence factors in the classical sense are those involved with antibiotic resistance. They are not central to pathogenesis in experimental disease, but are critical when discussing the clinical treatment of melioidosis. *B. pseudomallei* is intrinsically resistant to many antibiotics, greatly limiting therapeutic options and often resulting in inappropriate empiric treatment. This resistance in conferred by several mechanisms common to many bacteria, which includes cellular exclusion, enzymatic degradation, target modification or elimination, and active efflux [258]. Outer membrane permeability is an important mechanism of antibiotic resistance in closely related species, particularly as part of a synergistic relationship with efflux pumps, but has not yet been directly studied in *B. pseudomallei* [258]. Enzymatic degradation via the *penA* encoded Class A β-lactamase is one mechanism of resistance to unpotentiated penicillins and first and second generation cephalosporins [259,260]. Mutations in the *penA* gene have also resulted in isolates that are resistant to ceftazidime or penicillins potentiated with clavulanic acid [261]. OXA-57, a class D beta-lactamase has also been identified in *B. pseudomallei* [262], along with five other β-lactamases (Class A, B, or D)

that have been suggested by genomic analysis [263]. Deletion of the gene encoding a penicillin-binding protein 3 also conferred resistance to ceftazidime in clinical isolates *B. pseudomallei* [264].

At least 10 efflux pumps of the resistance-nodulation-cell-division (RND) family have been discovered in *B. pseudomallei*, though only seven of the pumps are likely to function in drug efflux [265]. Three of the drug efflux pumps have been described in terms of their function/substrates: AmrAB-OprA effluxes aminoglycosides and macrolides [266]; BpeAB-OprB from Bp KWH effluxes the aminoglycosides gentamicin and streptomycin, the macrolide erythromycin, and the dye acriflavine [267], while BpeAB-OprB from Bp1026b effluxes macrolides, fluoroquinolones, tetracyclines, acriflavine, and (weakly) chloramphenicol [268]; and BpeEF-OprC effluxes chloramphenicol and trimethoprim [269]. BpeAB-OprB from Bp KWH has additionally been described to play a role in quorum sensing and dependent *in vitro* virulence factors such as siderophores, phospholipase C, and biofilm formation [270,271]. However, just as differences in drug efflux for BpeAB-OprB KWH and 1026b, the dependence of quorum sensing and virulence in 1026b was not dependent on BpeAB-OprB [268]. The reason for these strikingly different findings remains unclear. While attenuation of *in vitro* virulence has been associated with mutations of some drug efflux pumps in *B. pseudomallei*, no *in vivo* study has found attenuated virulence [266], which is strongly supported by the fact that clinical (and therefore virulent by definition) isolates show similar efflux pump mutations [272].

The ability of *B. pseudomallei* to produce biofilms does not appear to function as a virulence factor in experimental infection [273]. However, the production and presence of biofilms plays an important role in *in vitro* and possibly *in vivo* clinical antibiotic resistance [274,275].

1.10) Host Immune Response and Intracellular Survival Mechanisms

The host-pathogen interactions that are important to the pathogenesis of melioidosis and immune response to disease are still relatively poorly understood. Many studies have been conducted *in vitro* and *in vivo* with various combinations of genetically manipulated bacteria, cell lines, and knockout mice. A few studies have also examined the host response on a genome-wide scale, which has provided important insights, but an integrated holistic understanding of the process is still lacking [238,276-278]. The following examination of the host immune response in melioidosis reviews molecular and cellular events in the host-pathogen interactions that define the host immune response. The effects of a diabetic disease state are also highlighted in regards to the immune defects mentioned in Section 1.7a since melioidosis is most often a disease associated with a pre-existing degree of immune compromise. In order to better understand this interplay, diabetic melioidosis patients as well as experimental models of diabetes and melioidosis have been examined on a limited basis. This area has also been well reviewed by Cheng and Currie in 2005 [14] (though a substantial amount of relevant research has been conducted since 2005) and Wiersinga and van der Poll in 2009 [279].

1.10a) Innate Immunity

Complement has been shown to readily bind to *B. pseudomallei* through the alternative pathway, though neither opsonization, phagocytosis, and polymorphonuclear cell and oxidative burst, nor membrane attack complex deposition resulted in bacterial killing [280]. A more recent study has further demonstrated that the capsule of *B. pseudomallei* limits the binding of C3b to the bacteria, thereby limiting the complement cascade, phagocytosis, and supporting the survival of bacteria in the blood stream [227]. *B. pseudomallei* has been found to be resistant to some antimicrobial peptides as well [281].

Neutrophils have been shown to be critical to the immune response in melioidosis [108], even though *B. pseudomallei* induces a relatively low level of IL-8 production [282]. The recruitment of neutrophils is dependent on MyD88 signaling, as seen by an *in vitro* reduction in TNFα production and *in vivo* reduction in bacterial clearance and accelerated mortality (with no effects on neutrophil phagocytosis) in MyD88 knockout cells and mice [283]. These findings suggest a greater risk to diabetics given their reduced chemotactic and killing capacity of neutrophils. This is supported by an *in vitro* study of the response of diabetic and healthy control derived neutrophils to *B. pseudomallei*. The study found that phagocytosis of *B. pseudomallei* was significantly decreased in poorly controlled diabetics and *B. pseudomallei* stimulation of neutrophils decreased migration in response to IL-8 [135].

IFNγ, produced by NK cells and bystander CD8+ T Cells [284], is also essential for host resistance to *B. pseudomallei* [285], the production of which is also significantly decreased in diabetics [134]. Melioidosis patients show significant elevations of IFNγ and its inducing cytokines, IL-12p40, IL-15, and IL-18, and IFNγ related chemokines, CXCL10 (IP-10) and CXCL9 (MIG) [286,287]. Melioidosis patients with higher IL-18 and IL-18 binding protein were shown to have higher mortality, and IL-18 knockout mice have additionally been shown to have accelerated mortality when infected with *B. pseudomallei* [288]. Elevations of other cytokines such as IL-1β, IL-6, IL-8, TNFα, IL-10, and TGF-β have all been noted in melioidosis patients, with IL-6 concentrations being the most predictive of mortality [289-291]. Several more pro-inflammatory (IL-1β, IL-6, IL-15, IFN-γ, TNF-α, TNF-β, CCL3 (MIP-1α), CCL4 (MIP-1β)), and anti-inflammatory (IL-4, IL-10, IL-1 receptor antagonist, and TNF receptor 1) molecules are significantly upregulated in the leukocytes of human melioidosis patients and have been identified by mRNA expression profiling and significantly correlated with mortality (IL-1β,

IL-1 receptor antagonist, CCL3, NF-κB1, NF-κB1A, and TNF receptor 1) [292]. Excess cytokine production/dysregulation and resultant immune pathology are important components of the overall pathogenesis of melioidosis [219]. The signaling for this cytokine production and much of the innate immune response is mediated through pattern recognition receptors such as TLR1, TLR2, TLR4 and CD14 co-receptor, and TLR5, as well as and nucleotide oligomerization domain (NOD)-like receptors (NLRs) NLRP3 and NLRP4, which have all been implicated in melioidosis through *in vitro* and/or *in vivo* studies [7,236,237,293-296].

Macrophages are essential for host resistance to *B. pseudomallei* [297,298] and are the target of several virulence factors of *B. pseudomallei*. The central role of the macrophage is to phagocytose and kill *B. pseudomallei* with reactive nitrogen and oxygen intermediates (RNI and ROI) when activated by IFNγ [298-300] and/or lymphocytes [301]. *B. pseudomallei* uses multiple virulence factors to inhibit the production of nitric oxide (RNI), including: LPS capsule limiting/modulating the strength of the cellular response [302]; upregulation of host arginase 2, which competes with nitric oxide synthase 2 (NOS2) for arginine [238]; induction of SOCS-3 and cytokine-inducible Src homology 2-containing protein (CIS) expression, which interferes with IFNγ production and therefore the downstream expression of NOS2 [303-305]; activation of sterile-α and armadillo motif-containing protein (SARM), which is a negative regulator of the MyD88-independent (TRIF-dependent) pathway involved in the production of IFNβ and the downstream expression on NOS2 [306-308]; and TssM, which inhibits NF-κB and type 1 IFN pathway activation [309]. The global regulatory factor RpoS has also been found to be crucial for NOS2 activation as well as multinucleate giant cell formation [310]. Many more potential genome-encoded factors with anti-macrophage activity have been reported from a genome-wide analysis of *B. pseudomallei* [311], as well as the transcriptional adaptation associated with the

survival of *B. pseudomallei* in macrophages, which will further define relevant bacterial factors and their role in pathogenesis [312].

Beyond the intracellular subversion of macrophage defenses, *B. pseudomallei* can induce programmed cell death signaled through NLRs and caspase-1dependent pyroptosis as well as apoptosis as a bacterial-driven mechanism of escape to avoid killing by inducing the death of the macrophage [7,313,314]. Activation of apoptosis is another function of RpoS, which has been shown to be essential for the regulation of macrophage death [315].

Monocytes from diabetic melioidosis patients also have been shown to have decreased phagocytosis [316] and nitric oxide production [317] in response to *B. pseudomallei* compared to healthy controls (no assessment of healthy diabetic patient monocyte function were made such that diabetic and melioidosis effects could not be separated). *B. pseudomallei* was found to have increased survival inside peritoneal elicited macrophages and induce significantly less production on IL-1β, IL-12, and TNF-α from chronically diabetic mice compared to healthy controls [138].

Autophagy is another innate immune mechanism induced in host epithelial cells and macrophages in response to infection with *B. pseudomallei* [7]. Stimulation of autophagy reduces intracellular survival of *B. pseudomallei*, but it is generally resistant to autophagic killing. The mechanism of this resistance appears to be an active process (not present in response to killed bacteria) dependent on an intact T3SSs and BopA [318,319]. Autophagy of *B. pseudomallei* does not involve the canonical formation of an autophagosome, but is achieved by LC3-associated phagocytosis (LAP), which involves recruitment of LC3 (a protein marker of autophagy) to phagosomes containing bacteria [320]. Both canonical autophagy of free bacteria within the cytoplasm and LAP are generally ineffective at killing *B. pseudomallei*. A suggested

mechanism for bacterial evasion of autophagy is the disruption of host ubiquitin-tagging by the deubiquitinase activity of TssM [218,309].

1.10b) Adaptive Immunity

The majority of epidemiologic evidence suggests that increased risk for melioidosis is associated with defects in innate immunity. Adaptive immune deficits have been described in diabetic patients [321-324], but appear to be secondary to defects in innate immunity regarding susceptibility to melioidosis. This is most striking illustrated by the finding that there is no increased risk of melioidosis seen in HIV (such as is seen with tuberculosis) [145,146]. Humoral and cell-mediated adaptive immune responses have been characterized in melioidosis [325-333], and are involved in the resolution of disease. However, the overall role of adaptive immunity is poorly understood as again highlighted by the fact that HIV infection does not appear effect the presentation of outcome of melioidosis in co-infected individuals [145]. Previous exposure and/or infection to *B. pseudomallei* results in antibody and cell-mediated immunity, including the generation of memory T cells [328,334], but is generally not associated with protection from subsequent re-infection [335]. However, one study did recognize statistically lower mortality in melioidosis patients that had previously had melioidosis [176].

1.11) Diagnosis

The protean signs and highly variable presentations of melioidosis make the clinical diagnosis of melioidosis virtually impossible without confirmatory laboratory testing. Positive culture of *B. pseudomallei* is still seen as the 'gold standard' diagnostic test since it is 100% specific and has a 100% positive predictive value since the bacteria is never found in healthy individuals [336]. The sensitivity and negative predictive value of culture are notably lower –

around 60% – because of low numbers of or intermittent presence of bacteria, insufficient sampling, or the previous administration of therapeutics [336]. This limitation is not unique to culture based techniques, but can be important in any diagnostic test dependent on the detection of bacterial antigens. The diagnostic utility of culture varies based on the type of sample used. Blood culture is the most common diagnostic test performed, with most reports documenting approximately 50% of patients as blood culture positive. Urine culture is positive in 21 to 28% of melioidosis patients [26,337]. Sputum culture has been found to be positive in 78% of patients in one study, while culture of throat swabs is generally less sensitive and more variable, with positive cultures in 36 to 61% of patients [185,337,338]. Pus is the least frequently collected/tested sample in melioidosis patients, but when collected it was positive in 82% of patients in the study [185,337].

Besides the lower sensitivity, a significant limitation of culture-based methods is the time required to identify the bacteria to species. Culture requires at least 24 hours and more typically 48 hours with additional time required to perform confirmatory tests such as antibody agglutination [339,340], immunofluorescence [341], gas-liquid chromatography analysis of bacterial fatty acid methyl esters, or PCR [342,343]. Substrate utilization panels, such as the API 20E, API 20NE, and Vitek 1, have shown mixed results in their ability to reliably identify *B. pseudomallei*, and have fallen out of favor in certain areas [14,342,344]. The time for diagnosis is a serious limitation for culture-based techniques since half of deaths from melioidosis occur within the first 48 hours, most empiric antibiotic therapy is ineffective for *B. pseudomallei*, and inappropriate therapy has been shown to be significantly associated with mortality [72,80]. Alternate methods of blood culture have been developed with a goal of decreasing the time to diagnosis, but typically do so at the cost of decreased sensitivity [345].

The most rapid methods for definitive diagnosis are tests based on the detection of antigen. Several experimental ELISA and immunofluorescence-based methods have been developed and were reviewed by Cheng and Currie in 2005 [14]. The only one to gain widespread use in a clinical setting is the monoclonal antibody latex agglutination test, which is only used in conjunction with culture to detect *B. pseudomallei* in blood culture fluid. The other tests appear to have failed to progress to diagnostic use because of issues of availability, ease of use, or poor performance during field testing [14,340]. New antigen-based tests continue to be developed because of the advantages of rapid diagnosis of active disease that is independent of issues of immunoprevalence [346], which is a significant issue for serological testing methods.

Direct immunofluorescent microscopy of clinical samples is one of the most rapid tests possible for the diagnosis of melioidosis and performed well compared to culture with a sensitivity of 73% and a specificity of 99% [347]. However, the utility of this technique is limited throughout much of the endemic range of *B. pseudomallei* because of the requirement for advanced microscopy facilities. An immunohistochemical method for detecting *B. pseudomallei* in formalin fixed tissues, is more accessible for most laboratories [348], but is not in widespread use.

Multiple molecular diagnostic techniques, including conventional PCR, quantitative real time PCR (qPCR), multiplex qPCR, multiplex qPCR paired with electrospray ionization mass spectrometry, and DNA microarrays have been developed for the identification of *B. pseudomallei*. Some of this work has been devoted to improving diagnostics in melioidosis cases [349-354], while a great deal has also been focused on the rapid detection of biothreat agents, especially for differentiating the closely related *B. mallei* and *B. pseudomallei* [355-362]. Recently, several different qPCR targets were assessed for their ability to detect *B. pseudomallei*

in clinical samples, with the type three secretion system open reading frame 2 (TTS1-*orf*2) target (originally described by Novak *et al*. [354]) showing the greatest analytical specificity, a limit of detection of five genome equivalents, a diagnostic sensitivity of 80% and a diagnostic specificity of 100% [351]. However, the greatest limitation of this diagnostic technique is that it is inaccessible for many medical centers throughout the endemic range.

The most widely used non-culture-based serologic test remains the IHA, which is performed essentially the same as its original description for the diagnosis of melioidosis in goats in 1965 [363]. The test is inexpensive and easy to perform, which allows for widespread use. However, there are several notable limitations of the test. The strains of *B. pseudomallei* used to generate whole cell antigens are not standardized, such that accurate comparisons of titers determined at different laboratories is not possible [14]. The sensitivity of the test for patients at admission is low, with only 51 to 57% of Australian patients reported to present with a titer ≥1:40 [335,364]. The majority of seronegative melioidosis patients do seroconvert, but 26 to 32% of patients with culture confirmed melioidosis remain seronegative [335,364]. Conflicting results have been found regarding the cross-reactivity between *B. pseudomallei* and *B. thailandensis* in the IHA, which could affect assay specificity [212,213]. It is increasingly believed that the higher seropositivity seen in Thailand is more a result of repeated environmental exposure to *B. pseudomallei*, rather than antibodies to *B. thailandensis* [335]. The most recent assessment of the overall sensitivity, specificity, positive predictive value, and negative predictive value of the IHA were 69.9%, 83.9%, 87.5%, and 64.3% [336].

Several other serologic methods have also been examined for the diagnosis of melioidosis in an effort to develop a test with a better diagnostic accuracy than the IHA. The only test to predate the IHA for the diagnosis of melioidosis is the complement fixation test [365]. While the

test performed well in experimental studies, lower specificity was observed in human patients, particularly cross reaction with *Pseudomonas aeruginosa* [366,367]. More recent serologic techniques include primarily ELISAs [368,369] and immunochromatographic test (ICT) kits [370-373]. These test typically perform as well or better than the IHA, but all of these tests are significantly limited by low negative predictive values and therefore have limited ability to rule out disease [336]. However, recent re-examination of ELISA techniques using Bayesian latent-class models indicated that these assays have better sensitivity and specificity than previously reported such that it may be possible to develop them into useful diagnostic tests for melioidosis [374]. Unfortunately, the high background seroprevalence to *B. pseudomallei* greatly lowers the diagnostic utility of serologic tests in endemic regions, though they may be more useful in non-endemic regions with low seroprevalence for travelers or in the event of suspected act of malicious release [369,370]. Advancing protein microarray technology may aid in the development of serodiagnostic tests that can help distinguish environmental or background exposure from true disease [375,376].

1.12) Therapy

1.12a) Current Recommendations

The current treatment recommendations for melioidosis have been well summarized by Wuthiekanun and Peacock (2006) [377], Cheng (2010) [378], and Inglis (2010) [379]. Cheng also provides an excellent review of the clinical trials that formed the basis for the current recommendations [378]. Therapy typically consists of an initial 10 to 14 day intensive phase followed by a prolonged (three to six month) eradication phase. Cutaneous and pediatric melioidosis cases have been treated successfully with oral antibiotics alone and shorter durations

of therapy [64,201,205]. The intensive phase consists of intravenous ceftazidime, imipenem, or meropenem [380]. Adjunctive therapy with granulocyte colony-stimulating factor (G-CSF) is used in some Australian cases based on the evidence that neutrophil function is impaired in several subsets of melioidosis patients and a retrospective finding of a significant reduction in mortality from septic shock in melioidosis with the use of G-CSF [381]. However, a prospective, randomized, controlled trial of G-CSF in Thailand demonstrated a longer duration of survival, but no reduction of mortality such that its use is not supported in Thailand [6,382]. The addition of trimethoprim-sulfamethoxazole in the intensive phase has also been advocated for use in deep-seated infections [379], but this has not been supported by clinical trials [383]. A clinical trial assessing the efficacy of ceftazidime vs. meropenem is ongoing in Thailand [6].

The eradication phase initially consisted of an oral four drug combination of trimethoprim-sulfamethoxazole, doxycycline, and chloramphenicol. Clinical trials have demonstrated that chloramphenicol and doxycycline can be removed from the treatment protocol, leaving just trimethoprim-sulfamethoxazole dosed on a weight-based regime, with no adverse effects on mortality and significant improvements in patient compliance and reductions in side effects [384-386]. Amoxicillin-clavulanic acid is an alternative eradication therapy for patients allergic to sulfonamides [378].

1.12b) Experimental Therapeutics and Vaccines

The limited armamentarium of effective antibiotics for the treatment of melioidosis, the presence of isolates that are resistant to some of these antibiotics, high cost and long duration of therapy, and persistence of relapsing disease in a percentage of patients with appropriate treatment all highlight the need for novel therapeutics [387]. Evaluation of therapeutics can be conducted *in vitro*, but *in vitro* efficacy frequently does not equate to *in vivo* efficacy in mouse

models or human clinical trials [387]. Of the limited studies conducted in mice, the focus is generally restricted to therapy in an acute inhalational model, with drastic reductions in efficacy associated with delays in the implementation of therapy post-infection [388-391].

Several novel therapeutics have been evaluated that show excellent *in vitro* killing of *B. pseudomallei*, which are hoped to translate into *in vivo* efficacy. The drug BAL30072 belongs to the group of monocyclic beta-lactam (sulfactams or monosulfactam) antibiotics. BAL30072 gains entry to bacterial cells through molecular mimicry of siderophores and then functions as a powerful inhibitor of class C β-lactamases and bacterial penicillin binding proteins. BAL30072 is also resistant to hydrolysis by bacterial class B metallo-β-lactamases [392-394]. Farnesol, a sesquiterpene alcohol, appears to disrupt cell wall synthesis, and in doing so works to potentiate β-lactam antibiotics [395,396]. Cathelicidin antimicrobial peptides LL-37 and LL-31 act through membrane disruption and show *in vitro* efficacy against both planktonic and biofilm associated *B. pseudomallei* [395,397]. Nitric-oxide based antibiotics (hydroxyurea, spermine NONOate, and DETA NONOate), have been examined for their notable ability to kill *B. pseudomallei*, including persistent bacteria, in a concentration and time dependent fashion under aerobic and anaerobic conditions [398]. While these compounds represent a significant pool of new candidate therapeutics for *B. pseudomallei*, their ultimate utility will have to be determined by their *in vivo* function.

The anti-diabetic drug glyburide was incidentally found to decrease melioidosis associated mortality in a study examining if increased susceptibility to melioidosis in diabetics was associated with increased mortality. The study did find that diabetics had lower mortality from melioidosis, but that this effect was because of the use of glyburide [399]. Glyburide is a potassium ATP-channel blocker and broad-spectrum ATP-binding cassette transporter associated

with immunomodulatory effects downstream to its inhibition of inflammasome formation, but it remains unclear exactly how this enhances survival in diabetic melioidosis patients and if glyburide would be beneficial in non-diabetic patients [399].

1.12c) Experimental Vaccines and Immunotherapy

While only a handful of novel antimicrobial compounds have been identified as potential therapeutics for melioidosis, a much wider variety of antigenic molecules and vaccine types have been identified and preliminarily evaluated for protection against melioidosis. Many of the vaccines are effective at delaying and/or preventing mortality (for the duration of the experiment), but no experimental vaccine to date has achieved the ultimate goal of sterilizing immunity in the face of a robust challenge. Despite the importance of melioidosis as a naturally occurring disease, nearly all vaccines developed are targeted towards biodefense models, which limits the ability to assess the utility of these vaccines for the prevention of melioidosis in endemic regions [400]. A full examination of experimental vaccines is beyond the scope of this review, but several articles provide excellent overviews of strategies employed for the development of a vaccine for melioidosis, as well as summarize the various live attenuated, whole killed, subunit, plasmid DNA, and dendritic cell vaccines that have been evaluated in murine models [400-402]. In addition to the 29 vaccines reviewed by Peacock *et al.* [400], one recent study examined the potential of a type IV pilus protein subunit vaccine, which did not confer any protection to vaccinated mice [403]. Another study using a live attenuated relA spoT double mutant provided significant protection from mortality but did not achieve sterilizing immunity [254].

Immunotherapy to enhance the innate immune response as a preventative measure or post-exposure treatment with CpG oligonucleotides (ODN), often in association with cationic

liposomes, has also been evaluated in murine models of melioidosis. The mechanism of action of CpG ODN appears to be to enhance or prime the innate immune response, which more effectively limits infection by *B. pseudomallei* [404]. The combination of CpG ODN with cationic liposomes has been shown to prolong this immunostimulatory effect [405]. Several studies have examined the role of CpG ODN in immunotherapy and also as a potent vaccine adjuvant for melioidosis, which are reviewed by Estes *et al.* [387].

1.13) Recurrent Melioidosis

Despite prolonged antibiotic therapy, the most common complication of surviving melioidosis patients is recurrent melioidosis, which occurs in 3 to 23% of cases [8,63,74,80,84,406-411]. Recurrent melioidosis must be subdivided into cases of relapse or re-infection. Early studies relying on data primarily from the time prior to the advent of ceftazidime therapy demonstrated that 92% of recurrent infections were from relapse [411]. More recent studies of recurrent melioidosis have found that 65 to 75% are caused by the same strain (relapse) and 25 to 35% are new strains (re-infection) [408,410,412]. It has also been suggested that the apparently high incidence of re-infection could partially be the result of simultaneous infection with several strains of *B. pseudomallei*, which may not be recognized at the initial typing and therefore appear as novel infecting strains at the time of recurrent disease [413].

Relapse infections are often attributed to poor adherence to antibiotic therapy [8]. Oral antibiotic choice and duration of therapy (≤ 8 weeks), positive blood culture, and multifocal distribution were all significantly associated with a greater risk of relapse [409,410]. Conflicting results have been found regarding the significance of intravenous antibiotic choice and its relation to relapse [409,410]. No differences in demographic data or clinical presentations were

noted for patients with melioidosis relapse versus primary melioidosis patients [410]. The patterns of organ involvement for relapse are generally similar to the pattern of organ involvement at initial presentation [409,410]; however, the same was true for cases of re-infection, suggesting that host factors are important in determining patterns of disease presentation [410]. The incidence of re-infection is much higher than the incidence of primary infection [412]. Even though no risk factors for re-infection have been defined, the epidemiologic evidence suggests that they must exist and likely relate to individual immune function or exposure/occupational factors [407,410,412]. The bacterial mechanisms contributing to the development of recurrent or latent melioidosis are generally unknown. Lipopolysaccharide type has been identified as bacterial factor that may be involved in the development of relapse infections [414]. Recent studies have employed deep sequencing to examine the genetic basis of evolution of *B. pseudomallei* in recurrent infection [415], as well as a proteomics-based approach to examine differential expression of bacterial proteins in primary and relapsing melioidosis [416]. Once the mechanisms associated with relapse are better defined, novel therapeutics to target these areas may help to prevent the occurrence of relapsing melioidosis.

A small percentage of disease (3-4%) is attributed to re-activation of latent infection [8,406]. The divisions between latent infection and relapse are poorly defined, but the existence of a latent form of melioidosis is well recognized [417,418], even though there is no way to currently detect latent infection until it manifests as reactivated disease. Latent infections are believed to be rare because of the strong seasonal pattern of melioidosis cases [52,406]. Several notable cases of very long periods of latent infection have been reported [147-149], but little is known about the bacterial or host factors involved in latency because of the rarity of latency in

human patients (as well as an inability to diagnose and study the latent state) and the fact that there are no animal models of latent infection [418].

1.14) Pathology

Despite the high mortality rate in melioidosis, historically and currently, throughout much of its endemic range, there are relatively few reports documenting the gross pathology or histopathology of human melioidosis. This is in large part a result of the cultural and religious prohibitions of postmortem examinations in Southeast Asia. The largest series of pathological cases remains Whitmore's original description of 38 cases in 1913, in which he could only examine the pathology of disease in the abandoned wastrels of society [9]. While this certainly created a selection bias and an acknowledged limited understanding and skewed perspective of the illness, the majority of his observations of the gross pathology remain consistent with subsequent reports as well as the current understanding of disease. Given the frequency and prominence of pulmonary lesions, the disease was initially believed to be a primary pulmonary infection. However, as additional cases were observed, the involvement of multiple organs, absence of pulmonary lesions in some cases, and the notable involvement of the spleen suggested that disease was a primary septicemia [9]. Splenic involvement was typically characterized by an enlarged, soft spleen from which bacteria were easily cultured. Small splenic miliary abscesses were noted in some cases as well as less frequent abscesses in the liver and kidneys [9]. The abscess structure was similar in all organs, but was best described from pulmonary lesions as an area of consolidation with a central area that is pale, soft, and cheesy surrounded by an outer zone of congestion and small hemorrhages [9].

The predominant source of additional gross pathology come from military personnel that succumbed to melioidosis during or shortly after serving in endemic regions during the Vietnam

War, though earlier cases were reported from soldiers in Burma during World War II and the Malayan War [419]. Soldiers represent a unique group of melioidosis patients since they are typically younger and without serious predisposing conditions, but can have high occupational/environmental exposure risk. Despite the current evidence that patients without known risk factors are more likely to have less severe disease, the majority of patients presented with acute, severe disease [420-422] (though this observation is likely skewed by the fact that these are reviews of fatal cases). A group of four soldiers were predisposed to acute, systemic, fatal disease following extensive burns immediately prior to exposure to soil and dirty water [423]. Another series that described three fatal cases out of ten total, noted diabetes, obesity, and excessive alcohol consumption in several of their melioidosis patients [419]. One autopsy study of six cases originating in Thailand has also been reported [424].

In one peracute case, no gross lesions were observed, while acute cases had pulmonary lesions and often hepatic and splenic lesions as well [423]. Deaths in subacute cases generally exhibited a wider distribution of gross lesions than seen in acute cases [420], which included the following organs: pancreas, kidneys, adrenal glands, lymph nodes, seminal vesicles, prostate, skin, joints, and pericardial space [419-421,423,424]. Gastrointestinal signs (diarrhea) were often present, but gastrointestinal lesions were never observed [419,422]. Visceral lesions are typically whitish yellow to tan, without the hemorrhagic border observed in pulmonary lesions. It was recognized that lesions could easily present it any location based on the septicemic nature of disease.

While the descriptions of gross lesions are very consistent among reports, histopathologic findings tended to vary to a greater degree. Microscopic lesions of coagulative and single cell necrosis are rarely reported but are found in a variety of organs [422,424]. Histologic

examination of gross lesions generally describes a central area of inflammation with extensive cellular destruction and karyorrhexis [421], though reports varied on reporting a predominance of neutrophilic or histiocytic infiltrates [422-424]. In some reports, this central area was surrounded only by a zone of hemorrhage with or without degenerate histiocytes with the appearance of atypical giant cells that were not consistent with Langerhans' or foreign body giant cells [423,424]. The presence or absence of a zone of hemorrhage appeared to vary in association with the affected organ. Lung lesions were noted to have zones of hemorrhage in all reports, while hemorrhage in solid organ lesions was not observed in two studies [421,422], only the kidneys in one series [420], and in association with all lesions regardless of organ in two studies [423,424]. Despite the consistent presence of hemorrhage, vasculitis or vascular lesions were observed infrequently or not at all [422,425]. Fibrin thrombi associated with disseminated intravascular coagulation was noted in five of six acute fatal cases, which also had evidence of hemorrhage that was not necessarily associated with lesions [424].

Additional layers surrounding the necrotic center before the zone of hemorrhage have been described as well, which often include lymphocytes, plasmacytes, histiocytes, fibroblasts, epithelioid or epithelioid-like cells, and giant cells [420-422,425]. The presence of true giant cells or atypical giant cells, epithelioid or epithelioid-like cells, palisaded macrophages, and fibrosis varied by report, but were most common in chronic lesions. There was not a firm consensus as to whether lesions should be classified as true granulomas or just granuloma-like [420,422,425]. Chronic or acute-on-chronic lesions were more common in biopsy samples from non-fatal cases and rarely had histologically visible bacteria, even with Brown-Hopps staining [422,425]. This contrasted many of the fulminant, acute fatal cases where bacteria were easily visible and were also noted to form globi [423,425]. It should also be noted that regardless of

clinical presentation (acute, subacute, or chronic), lesions in a patient often consisted both acute necrotizing and chronic granulomatous forms at same time [420].

1.15) Animal Melioidosis

1.15a) Natural Disease

B. pseudomallei exhibits a very wide host range, especially when compared to other pathogenic bacteria. The most striking comparison is the host restriction of *B. mallei*, which is a deletional mutant of *B. pseudomallei*, which is a primary pathogen largely restricted to equids under natural conditions [426,427]. The extent of the host range has been highlighted by outbreaks in zoological gardens affecting many different species. The spectrum of disease epizootiology and affected species is well reviewed by Sprague and Neubaur [428]. Region specific reviews of animal melioidosis have also been published for Australia [105] and Thailand [429], which both identified goats as the most frequently affected species.

Several reports have documented and described the clinical and pathological findings of naturally occurring melioidosis in domestic species. Dogs and cats were first reported to be naturally affected with melioidosis by Stanton and Fletcher in 1932 [430] and several subsequent case reports have provided further description of disease presentation [431-437]. Melioidosis has also been well-described in goats, sheep, and other livestock [40,438-452], which parallels disease in humans with the exception that most livestock tends toward chronic presentations of melioidosis with granulomatous lesions [420]. Acute presentations in livestock are typically associated with central nervous system signs, which are relatively common in Australia [439]. The description of natural disease in goats will be discussed in later chapters in comparison to the experimental disease characterized in the dissertation research.

Though the initial description of the indirect hemagglutination assay for melioidosis was first described in goats [363], there has been relatively little research into serodiagnostics and seroprevalence of antibodies to melioidosis in animals. Besides the IHA, complement fixation, microagglutination, and more recently an ELISA have been evaluated for the diagnosis of melioidosis in goats and other species [106,366,442,453-455]. One large serologic survey of animal sera in Thailand found that pigs, deer, and sheep were found to have the greatest incidence of seropositivity (~7%), while goats had the lowest seropositivity of 0.33% [454]. This clearly conflicts with the incidence of culture proven cases [429], and the basis for this discrepancy is unknown.

1.15b) Experimental Models of Melioidosis

The early experimental investigations by Stanton and Fletcher examined the susceptibility of rats, mice, guinea pigs, rabbits, sheep, goats, fowl, monkeys, and horses to infection with *B. pseudomallei* by inhalation/nasal instillation, inoculation, scarification, or feeding [430,456]. Miller *et al*. studied susceptibility in hamsters, ferrets, guinea pigs, rabbits, mice, white rats, and monkeys to intraperitoneal, subcutaneous, and inhalational infection [457]. These early studies provided little detail beyond host susceptibility, which was difficult to compare between studies because of strain variation, route of infection, and limited quantitation of infecting dose. Mouse, hamster, rat, and guinea pig models all have been developed further and used to elucidate many aspects of melioidosis pathogenesis [233,281,458-465].

Experimental infection of livestock has been conducted in chickens (intramuscular, IM) [466], horses (subcutaneous, SC) [430], cattle (SC) [439,467], pigs (intravenous, IV and intratracheal, IT) [468,469], sheep (SC, IM, IV, supraconjunctival, intranasal, IN, and oral, PO) [439,448], and goats (SC and intraperitoneal, IP) [470,471]. These studies were also variable

and many provided limited if any quantitative information about infecting doses. Horses and cattle tended to only develop localized abscesses, while disseminated disease was frequently observed in the other species. The range of presentation varied from acute to chronic, with some animals resisting infection completely, all of which varied by dose, route, and likely individual susceptibility. Neurologic disease was most commonly observed following the experimental infection of sheep. Non-human primate aerosol models in the marmoset, rhesus macaque, and African green monkey have also been developed recently [472,473]. Two reviews of animal models of melioidosis have also been published, summarizing the findings of the various small and large animal models examined [474,475].

The most widely used model species is the mouse, which has been a logical choice because of the availability of and ability to create many immunologic mutants, detailed genomic information, and the widest scope and variety of reagents and diagnostic capabilities. Given these possibilities, murine models have been used to study all aspects of melioidosis. The importance of individual (mouse strain) risk factors and route of infection for the development and outcome of melioidosis are two aspects of murine models that investigate particularly important, yet poorly understood, areas of melioidosis pathogenesis [6].

The incorporation of risk factors (such as diabetes) into murine models of melioidosis have largely been ignored [400], with the exception of a handful of recent diabetic mouse models [137-140]. The surrogate comparisons to examine individual susceptibility have instead been based on different inbred mouse strains, primarily BALB/c versus C57BL/6, with BALB/c mice being consistently more susceptible to *B. pseudomallei*, with much lower LD50s than their C57BL/6 counterparts [476]. Infection with *B. pseudomallei* also has been investigated to a much lesser extent in C3H/HeN, DBA/2, Taylor Outbred (TO), 129/SvEv,

SWISS, CBA, CD-1, Namru Albino, BDF1, and SCID mouse strains [459,475,477]. The relative susceptibility has been reported as CBA > 129/SvEv > BALB/c > DBA/2 > C3H/HeN >> (C57BL/6 and TO) [475].

The basis for increased resistance in the C57BL/6 mouse has been attributed to a more effective innate immune response (lower bacterial counts 12 h PI) and Th1 response [459]. Ulett *et al*. did not support the idea of differential polarized Th1 and Th2 responses in C57BL/6 and BALB/c mice leading to the different outcomes of infection with *B. pseudomallei*. More rapid and higher levels of IFNγ and TNFα in BALB/c mice suggested that there was not a deficiency of a Th1 response, but that overproduction of these cytokines lead to immunopathology and increased mortality [478]. The overproduction and or imbalance of cytokines/chemokines (IFNγ, TNFα, IL-6, GM-CSF, M-CSF, CCL2 (MCP-1), CCL4, CCL5 (RANTES), CXCL1 (KC), CXCL2/3 (MIP-2α/β), CXCL9, and CXCL10) and resultant immunopathology has been repeatedly found in the BALB/c to C57BL/6 comparison, but is complicated by the caveat that cytokine levels are closely associated with bacterial counts [479-482]. Therefore all cytokine differences between groups/strains must be interpreted in light of the bacterial organ burden, which is consistently higher in BALB/c mice [479]. When examined histologically, the lesions in BALB/c mice contain a much greater infiltration of neutrophils compared to C57BL/6 mice, which have a predominance of macrophages [479]. This implies an inability to contain the infection, which may be either the result or the cause of the exuberant cytokine response that is associated with higher bacterial organ burdens.

The majority of murine studies provide only very brief histologic descriptions of lesions and typically report only the presence of abscesses or granulomas [459]. A few notable studies have provided detailed histopathological descriptions of murine melioidosis. Lever *et al*.

describe the histologic lesions of acute (5 days) aerosol melioidosis in BALB/c mice [462]. Pulmonary lesions were that of focal, acute, necrotizing bronchopneumonia and alveolitis, with suppurative bronchial exudate and alveolar walls expanded by neutrophils and macrophages. Hepatic vacuolation, necrosis, and mixed inflammatory infiltrates, along with acute necrotizing splenitis were also commonly observed [462]. The lesions associated with a murine model of chronic melioidosis, low-dose intranasal infection of C57BL/6 mice, have also been described [483]. Chronic lesions were typified by pyogranulomatous lesions, in the lung, spleen, and liver, though granulomas without necrotic centers were also observed in these organs in infected mice [483]. Brief descriptions of lesions following enteral [200] and IP infection [477] report similar lesions in mice, with the additional findings of, focal gliosis and mild cardiac inflammation, or meningoencephalitis, respectively.

Despite the fact that many murine infection models of *B. pseudomallei* have been studied, relatively few specifically compare the effect of the route of infection or the question of pathogenesis beyond the effect of route on LD50 [482,484]. While aerosol, IN, SC, IV, IV, and PO/enteral infection routes have been investigated in murine models [94,200,459,462,481-487], a comparison of susceptibility across all routes is not possible because it has not been investigated in a uniform manner. Generally, IV, aerosol, IP, and IN infections are the most acute, followed by SC and then PO infection [482,484], though there is high variability based on the bacterial and mouse strains investigated. One study found that whole-body aerosol exposure eliminated the BALB/c and C57BL/6 strain difference [485], but a subsequent nose-only aerosol study supported the importance of genetic factors in the infected strain of mouse on susceptibility to *B. pseudomallei*, regardless of the route of infection [482].

Intranasal models are often used as a surrogate for true aerosol models, both of which have been primarily used to model malicious attack versus natural exposure. These infection models are the most frequently used because of the funding and research focus on biodefense and acute disease. Subcutaneous and oral/enteral infection models natural exposure, while IV infection is intended to model septicemia, though the bypassing of the initial exposure calls the validity of the model into question. Intraperitoneal infection is one that is convenient and has been used for many murine infection models, but does not model any natural route of infection. A detailed review of the routes of infection and studies examining them has recently been published by Warawa [475].

1.16) Rational for Current Studies

Even though the majority of research on the pathogenesis of melioidosis has been conducted in mouse models, a notable limitation of the murine (or any rodent) model system is almost uniformly ignored in melioidosis research. The limitation is that rodents do not appear to be naturally affected by *B. pseudomallei*, despite its wide host range. Melioidosis was initially mischaracterized as a disease spread by rats [488], but subsequent large studies in wild rodents rarely found evidence of disease or even seropositivity in rats, and neither in mice [489,490]. Therefore, while mice are, and likely will remain, the preferred small animal model of melioidosis, it is not possible to conclude that mice provide the best model of naturally-occurring human melioidosis [474].

The rational for the research that has culminated in this dissertation is the development and characterization of infection models of *B. pseudomallei* using a species naturally affected by melioidosis. Such a model can be assessed relative to disease seen in natural animal populations and provide a comparative basis for the study of the pathogenesis of disease in other

experimental models as well as humans, particularly in regard to the importance of the route of infection in disease presentation and outcome. Based on this rational, the hypotheses for the following experimental studies are: 1) goats can be experimentally infected with *B. pseudomallei* through aerosol and percutaneous exposure, producing models of human melioidosis; and 2) disease presentation and outcome will be influenced by the route of infection.

The goat (*Capra hiricus*) was selected as an optimal species based on the relatively high incidence of disease mirroring that of the human population in endemic regions. Outbreaks of disease have been observed in goats [491] similar to case clusters of disease seen in human patients. Caprine melioidosis from both natural and experimental infection can provide a unique opportunity to study aspects of melioidosis that have been inaccessible previously because of a lack of an adequate model system. This specifically applies to the development of chronic disease, the development of 'sterile' lesions (which could represent niduses of latent infection), and the apparent ability of goats to 'self-cure' [471]. Murine models of chronic and latent infection have been developed [474,483], but it is not clear if the disease states observed in mice are reflective of these states in human disease [418].

The goat can also provide a much needed second species for the testing of novel vaccines and therapeutics for the treatment of melioidosis [387] at a considerably lower cost than non-human primate models. The larger body size of goats also greatly facilitates clinical monitoring and therapeutic intervention on a human relevant scale. Goats are also an important agricultural species throughout much of the endemic range of melioidosis such that disease can cause both economic losses and pose a threat to public health. The knowledge gained from the future use of the caprine model in regards to improved diagnosis, preventive measures, and vaccination would be directly applicable to the management of these goat populations.

The following chapters detail the development and characterization of caprine aerosol and percutaneous infection models of melioidosis in terms of clinical, bacteriological, hematological, immunological, radiographic, and gross and histopathologic progression of disease.

CHAPTER 2: DEVELOPMENT AND CHARACTERIZATION OF A CAPRINE AEROSOL INFECTION MODEL OF MELIOIDOSIS[1]

2.1) Summary

Infection with *Burkholderia pseudomallei* causes the disease melioidosis, which often presents as a serious suppurative infection that is typically fatal without intensive treatment and is a significant emerging infectious disease in Southeast Asia. Despite intensive research there is still much that remains unknown about melioidosis pathogenesis. New animal models of melioidosis are needed to examine novel aspects of pathogenesis as well as for the evaluation of novel therapeutics. The objective of the work presented here was to develop a subacute to chronic caprine model of melioidosis and to characterize the progression of disease with respect to clinical presentation, hematology, clinical microbiology, thoracic radiography, and gross and microscopic pathology. Disease was produced in all animals following an intratracheal aerosol of 10^4 CFU delivered, with variable clinical manifestations indicative of subacute and chronic disease. Bronchointerstitial pneumonia was apparent microscopically by Day 2 and radiographically and grossly apparent by Day 7 post infection (PI). Early lesions of bronchopneumonia soon progressed to more severe bronchointerstitial pneumonia with pyogranuloma formation. Extrapulmonary dissemination appeared to be a function of pyogranuloma invasion of pulmonary vasculature, which peaked around Day 7 PI. Histopathology indicated that leukocytoclastic vasculitis was the central step in dissemination of *B. pseudomallei* from the lungs as well as in the establishment of new lesions. While higher doses of organism in goats can produce acute fatal disease, the dose investigated and resulting

[1] Chapter 2 was published as reference 492. Soffler C, Bosco-Lauth AM, Aboellail TA, Marolf AJ, Bowen RA (2012) Development and characterization of a caprine aerosol infection model of melioidosis. PLoS One 7: e43207.

disease had many similarities to human melioidosis and may warrant further development to provide a model for the study of both natural and bioterrorism associated disease.

2.2) Introduction

Despite its discovery nearly a century ago [1], melioidosis, or infection with *Burkholderia pseudomallei*, remains an emerging infectious disease of global importance [13]. Infection can occur through percutaneous inoculation, inhalation, or ingestion [14]. Resultant disease can range from an acute, fulminant septicemia to a silent infection that does not result in clinical disease for months to decades later [149]. The clinical signs produced by infection are protean in nature, making diagnosis difficult, especially in non-endemic regions where physicians are unfamiliar with the disease [493].

In northeast Thailand, melioidosis is currently the 3rd leading infectious cause of death, behind human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and tuberculosis [57]. Melioidosis appears likely to displace tuberculosis as the second most common cause of death due to infectious disease if the current trend in melioidosis mortality continues in that locale [57].

Mice have been the predominant host used for studying the pathogenesis and potential therapeutic interventions for melioidosis, and the availability of a large variety of inbred strains, immune function mutants, and immunologic reagents make murine models attractive for the study of this disease [474]. While a great deal has been learned from murine models, the authors are not aware of any papers documenting naturally occurring melioidosis in wild *Mus* spp., despite the very wide host range of animals affected by melioidosis [428]. Wild rodents, specifically rats, were initially believed to be central to the spread of *B. pseudomallei* to humans [488], but subsequent efforts to document disease, carriage, or antibody titers in rats found that

infection was exceedingly rare [489,490]. Given the highly variable nature of human melioidosis, the limited availability of clinical data from murine studies, and the limited publications documenting human pathology/histopathology [420,422,423,425], it is not possible to conclude that mice are a fully reflective model of naturally-occurring human melioidosis [474].

Goats are naturally affected by melioidosis and offer several potential advantages for use as an animal model of melioidosis, including a large body size allowing clinical monitoring and therapeutic intervention in a manner relevant to humans. Natural disease has been reported to occur in goats from Northern Australia [105,440-442,491] and Southeast Asia [429,438,443,444], two highly endemic regions for melioidosis [13], as well as in Aruba [445] and South Africa [446], which have sporadic incidences of disease. In addition to enzootic disease in goats in Northern Australia, outbreaks of melioidosis have also been documented [105,491]. Both acute and chronic melioidosis occur in goats [428], which encompass a wide variety of clinical signs and pathology [105]. Goats are one of the most frequently affected species [105,429], and comparatively speaking, there is a good documentation of the clinical signs and pathologic lesions associated with natural disease, which provides a basis of comparison to human disease as well as experimental models.

Two previous experimental models of caprine melioidosis have been published. Narita *et al.* (1982) used a goat isolate of *B. pseudomallei* passaged twice through hamsters prior to intraperitoneal (IP) or subcutaneous (SC) inoculation of goats with either 6.5×10^7 or 6.5×10^9 colony forming units (CFU) [470]. Animals infected IP developed septicemic disease of short duration ($\leq$ 10 d) with microabscesses found throughout the body, while animals infected SC showed no clinical signs other than fever for five days post infection (PI), but did develop

large abscesses in the spleen and lung that were found at necropsy (23 d PI) [470]. Thomas *et al.* (1988) used sheep, goat, or bird isolates of *B. pseudomallei* to infect goats SC with doses ranging from 90 CFU to 5 x 10^5 CFU [471]. Goats that received doses greater than or equal to 500 CFU developed fatal disease within two months PI [471]. Goats that received 225 or 90 CFU had signs that varied from no effect to fever and deteriorating condition that necessitated euthanasia [471]. Necropsy revealed abscesses that were most commonly found in the spleen, prescapular lymph node of the inoculated leg, and lungs [471]. Goats without clinical signs had no pathologic lesions when euthanized five months PI [471]. The great disparity between these two experimental models may have been a result of different virulence in the *B. pseudomallei* isolates, as suggested by Thomas *et al*. [471], but was also likely influenced by the different durations of the experiments and the time allowed for disease progression after subcutaneous inoculation.

In the 20 years since description of these original caprine models, which primarily focused on disease in goats and the potential impact on human health from raw milk consumption [471], the research landscape of *B. pseudomallei* and melioidosis has changed considerably. Significant advances have been made in the study of melioidosis, but there is still a great deal about *B. pseudomallei* infection that remains unknown. A greater understanding of pathogenesis and immune function can be achieved through comparative studies of different animal models and human disease. In pursuit of these goals, the objective of this study was to create a non-fulminant, subacute to chronic caprine model of aerosol-transmitted melioidosis. Such disease was readily produced, allowing for the description of the clinical, hematologic, microbiologic, radiographic, and pathologic features of caprine disease, adding a novel and useful model for the study of melioidosis.

2.3) Materials and Methods

2.3a) Bacterial Strain and Culture Methods

B. pseudomallei (Bp 4176/MSHR 511), isolated from an outbreak at an Australian goat farm [491] was generously provided by Dr. Apichai Tuanyok. Bacteria for infection were grown fresh in Muller-Hinton (MH) broth (M5887 Teknova, Hollister, CA, USA) at 37°C in air with constant shaking at 250 RPM. Bacteria were harvested in mid-log phase growth. Based on the OD600, the culture was diluted in phosphate buffered saline (PBS) to achieve a final concentration of 2 x 10^4 CFU/mL. The bacterial suspension used for infection was backtitrated in duplicate on MH agar plates incubated at 37°C in air.

Quantitative blood culture was performed using a pour plate technique modified from Simpson *et al*. [345]. Five milliliters of aseptically collected heparinized blood was mixed with 95 mL of molten (45°C) MH agar and poured into a 15 cm petri dish. Once solidified, the plate was incubated at 37°C in air. Plates were monitored for seven days before being declared negative. Colonies seen on blood culture plates were subcultured onto selective media (MH agar with 4 mg/L gentamicin [MH-gentamicin]) to verify gentamicin resistance and colony morphology consistent with *B. pseudomallei*. Nasal swabs, organ homogenates, and abscess material were also cultured on MH-gentamicin plates at 37°C in air and read at 96 h, 48 h, and 48 h, respectively.

All experiments using *B. pseudomallei* were performed in a biosafety level-3 facility.

2.3b) Aerosol Delivery Device

Bacteria were delivered as an intratracheal aerosol. The bacterial suspension was aerosolized using a LC Sprint® nebulizer (mass mean diameter 3.5 μm) and Vios® air

compressor (PARI Respiratory Equipment, Inc., Midlothian, VA, USA). The nebulizer was modified so that all exhaled air passed through a HEPA filter. The modified nebulizer was then connected to a cuffed endotracheal tube (size 10) for intratracheal delivery of the aerosol. A target dose of 1 x 10^4 CFU in 5 mL of PBS was selected based on pilot studies, which investigated a dose range of 10^4 – 10^8 (data not shown). Nebulization time was approximately 10 – 15 min. Total dose of bacteria delivered to the lungs was calculated as 10% of the bacteria placed in the nebulizer, a figure that was derived from pilot studies in which two goats were euthanized immediately after exposure and lung tissues samples representing all lung fields were homogenized and plated to determine CFU delivered per gram of lung tissue.

2.3c) Experimental Animals

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and every effort was made to minimize suffering. The protocol was approved by the Animal Care and Use Committee of Colorado State University (approval 11-2414A). Group size was determined subjectively based on pilot studies as the study was designed to be descriptive (vs. quantitative) in nature.

Twelve yearling Nubian-cross goats were obtained through private sale for use in this experiment. Goats weighed between 35 to 50 kg. There were seven males and five females. Animals were housed in an animal biosafety level-3 (ABSL-3) facility for the duration of the experiment. They had *ad libitum* access to water and were fed a complete pelleted feed twice daily. Goats were acclimatized to the facility for approximately one week prior to infection. Infection was performed (Day 0) under intravenous general anesthesia using xylazine

hydrochloride (premedication) and ketamine hydrochloride (induction/maintenance). Endotracheal intubation and nebulization was performed with the goats in sternal recumbency.

2.3d) Clinical Monitoring: hematology and clinical microbiology

Beginning on Day -4, the goats' attitude, appetite, and temperature were monitored daily for the duration of the experiment. Normal rectal temperature was defined as 38°C to 40°C. Four pre-infection complete blood counts (CBCs) (HemaTrue Hematology Analyzer, Heska Corporation, Loveland, CO, USA) were performed on each goat (except for goat 26, which had 3) to establish baseline hematologic values. Post-infection CBCs were performed on Days 1-7, 9, 11, 13, 16, 19, and 21.

Nasal swabs and blood cultures were collected on the same schedule as the complete blood counts, except that only one pre-infection sample was collected. For nasal swabs, both nostrils were sampled with a Dacron® fiber tipped plastic applicator swab (Cat. No. 14-959-90, Fisher HealthCare, Houston, TX, USA) and immediately streaked onto a MH-gentamicin agar plate. For blood culture, the collection site over the jugular vein was aseptically prepared with povidone iodine and ethyl alcohol, and 5 mL of venous blood was collected into a heparinized syringe.

2.3e) Thoracic Radiography

Three-view thoracic radiographs (right lateral, left lateral, and ventrodorsal) were taken under xylazine sedation for all goats prior to infection and on Days 7, 14, and 21. Radiographs of the extirpated lungs were also taken at the time of necropsy. Initial studies showed little to no radiographic change in the lungs on Day 2; therefore, this necropsy time point was not aligned with radiography. Radiographs were taken using a MinXRay® 100HF and Agfa® Computed

Radiography (CR) 43x35 CR MD 4.0 General Cassettes. During use within the ABSL-3, cassettes were triple sealed in 6 mil polyethelene bags (LAD 8555, Hillas Packaging, Inc., Fort Worth, TX, USA). The cassettes were processed with a CR 85-X digitizer and the images were processed with NX software.

2.3f) Euthanasia, Necropsy, Histology, and Organ Burden

Humane euthanasia of three goats with intravenous pentobarbital was planned for Days 2, 7, 14, and 21. One goat that was going to be euthanized on Day 2 died during recovery from anesthesia. Necropsies were performed on all goats. Based on organ involvement detected in pilot studies, the following tissues were collected into 10% neutral buffered formalin for histology: mandibular lymph node, retropharyngeal lymph node, tracheobronchial lymph node, mediastinal lymph node, lungs, heart, spleen, liver, kidney, adrenal, thyroid, brain, mesenteric lymph node, and testis or ovary. Samples were routinely embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for microscopic evaluation.

In organs with macroscopic abscesses, the abscesses were counted or estimated as a percentage of the total organ volume. Representative abscesses were cultured on MH-gentamicin agar to confirm positive growth of *B. pseudomallei*. In the absence of macroscopic abscesses, samples of the following organs were aseptically collected to calculate the organ burden (CFU/g): lung (cranial, middle, and caudal lobes of left and right lungs), spleen, liver, kidney, retropharyngeal lymph node, tracheobronchial lymph node, and mesenteric lymph node. Tenbroeck homogenizers were used to homogenize 0.5 g of each tissue sample in 2 mL PBS + 20% glycerol (limit of detection 50 CFU/g). Neat urine was also collected and cultured from each goat at the time of necropsy. The homogenate or urine (50 μL) was plated in duplicate on MH-gentamicin agar, and the remaining sample was frozen at -80°C. If the colony count was

above the limit of quantitation (>300 colonies/plate), the frozen homogenate was thawed and serial log dilutions were plated in duplicate.

2.4) Results

2.4a) Bacterial dose

Infection was performed on four separate days. The backtitration of the nebulized bacterial suspension ranged from 1.07 – 2.33 x 10^5 CFU. The delivered dose to individual goats was approximately one log lower and is reported in Table 2.1.

Table 2.1: Goats infected with *B. pseudomallei* and status at euthanasia.

Goat No.	Sex	Estimated Dose (CFU) *	Day PI Euthanized	Status at Euthanasia
13	M	1.2 x 10^4	2	Febrile, cough present
18	F	1.2 x10^4	2	Febrile
14	M	1.22 x 10^4	7	Subclinical disease
19	F	1.2 x10^4	7	Subclinical disease
27	M	2.3 x 10^4	7	Febrile
15	M	1.2 x 10^4	14	Febrile
20	F	1.2 x10^4	14	Subclinical disease
26	F	1.1 x10^4	14	Afebrile, intermittent cough
16	M	1.2 x 10^4	16	Afebrile, moribund, thick nasal discharge
21	F	1.2 x10^4	21	Subclinical disease
22	F	1.2 x10^4	21	Subclinical disease

* Estimated dose delivered to lungs calculated as 10% of the total amount of bacteria placed in the nebulizer.

2.4b) Clinical Monitoring

The median rectal temperature for all goats prior to infection was 38.9°C. Ten of 11 goats developed fever by Day 1 (median temperature 40.7°C), with the remaining goat

developing a fever by Day 2 (median temperature 41°C, n = 11). Fevers were variable, but peak temperatures were frequently seen on Day 6 (median temperature 40.7°C, n = 9) or Day 8 (median temperature 40.9°C, n = 6) after the Day 2 peak. Representative temperatures are shown in Figure 2.1. By Day 12 four of six goats had rectal temperatures within the normal range.

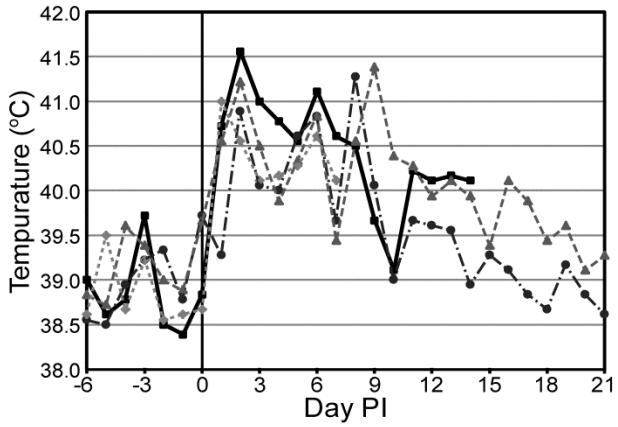

Figure 2.1: Temperatures from selected goats. Peaks in temperature are seen on Days 1 and 2 and between Days 6 and 9. Based on the temporospatial distribution of gross lesions, these peaks are likely associated with bacteremic dissemination events even though bacteremia was not detected with blood culture. BpG15 (■), BpG21 (●), BpG22 (▲), BpG27 (♦).

These four goats all returned to their pre-infection temperatures by the time of euthanasia on Days 14 or 21. Despite high fevers (up to 41.6°C), lethargy or decreased appetite was rarely observed.

Other than fever, the most frequent clinical sign recognized was cough, which was observed in seven of 11 goats. Cough was most frequently observed early after infection, Days 2 – 5. Nasal discharge was only observed in Goat 16, which developed on Day 11 and persisted until the time of euthanasia on Day 16.

2.4c) Hematology

Pre-infection hematology revealed a marked lymphocytosis in nearly all goats. The median pre-infection lymphocyte count for all goats over all independent samples was 12.8 x 10^3 cells/μL (range 3.5 – 23 x 10^3 cells/μL). This relatively high value was attributed to an epinephrine response, which is often seen in young, excited goats. The median pre-infection granulocyte and monocyte counts for all goats over all independent samples were 5.7 x 10^3 cells/μL (range 2.9 – 15.7 x 10^3 cells/μL) and 1.0 x 10^3 cells/μL (range 0.4 – 3.0 x 10^3 cells/μL). While some granulocyte and monocyte counts were elevated, no goat maintained a granulocyte or monocyte count above the reference range (1.2 – 7.2 x 10^3 cells/μL and 0.0 – 1.0 x 10^3 cells/μL respectively) for all samples. Variations were attributed to a cortisol response from handling. Pre-infection red cell parameters were generally unremarkable.

The only cell types to show a marked response post-infection were granulocytes. On Day 1 the median granulocyte count increased to 14.2 x 10^3 cells/μL and peaked on Day 2 at 16.7 x 10^3 cells/μL (n = 11). A second peak of 14.9 x 10^3 cells/μL was seen on Day 9 (n = 6). Representative changes in granulocyte counts can be seen in Figure 2.2.

2.4d) Clinical Microbiology

Nasal swabs were positive for *B. pseudomallei* for 3 of 11 goats on Day 1. This was very light growth, with three or fewer colonies per plate. At all other time points, positive nasal swabs were found only for goat 16 on Days 5, 11, 13, and 16; growth was light for this goat on Days 5 and 11 (less than 20 colonies) and heavy on Days 13 and 16 (dense mat of colonies).

Quantitative blood culture was negative for *B. pseudomallei* for all goats except for goat 16, which was positive on Day 13, with 1 CFU/5 mL, and on Day 16 with 19 CFU/mL.

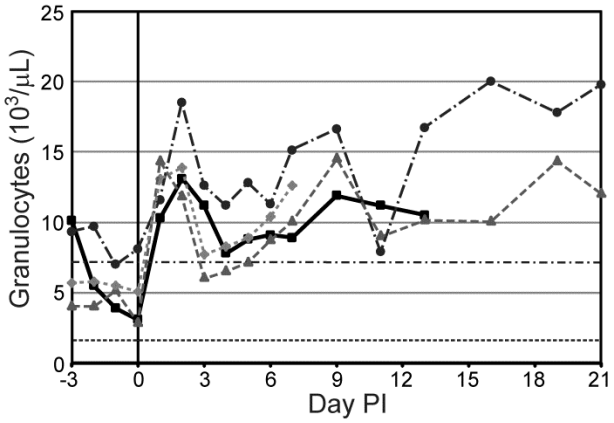

Figure 2.2: Granulocyte counts from selected goats. During the first half of the study, the granulocyte count appears to mirror the temperature trends seen in Figure 2.1. BpG15 (■), BpG21 (●), BpG22 (▲), BpG27 (♦), RR High (-·-·-)/Low (·····): reference range limits of normal caprine granulocyte count.

2.4e) Thoracic Radiography

There were no significant findings on pre-infection thoracic radiographs, except for goat 27, which had a moderate bronchointerstitial pattern; signs of respiratory disease were not evident in this goat prior to infection. Pulmonary disease was radiographically evident in all goats by Day 7 (n = 9), which ranged in severity from a mild to moderate bronchointerstitial infiltrates in the caudal lung lobes (Figure 2.3A) to severe bronchointerstitial infiltrates with variably sized nodules in all lung lobes (Figure 2.3B). Progressive nodular disease, with an increased number, size, distribution, and/or definition of nodules, was seen in all goats on Day 14 (n = 6). Progression of bronchointerstitial infiltrates, with an increase in severity and/or distribution, was seen in four of six goats. The remaining two goats had static bronchointerstitial infiltrates diffusely through all lung lobes. Of the two goats that survived to Day 21, both had static, moderate bronchointerstitial infiltrates diffusely. In one goat the nodules had increased in size, but the nodules in the other goat were static. Over the course of infection, the radiographic

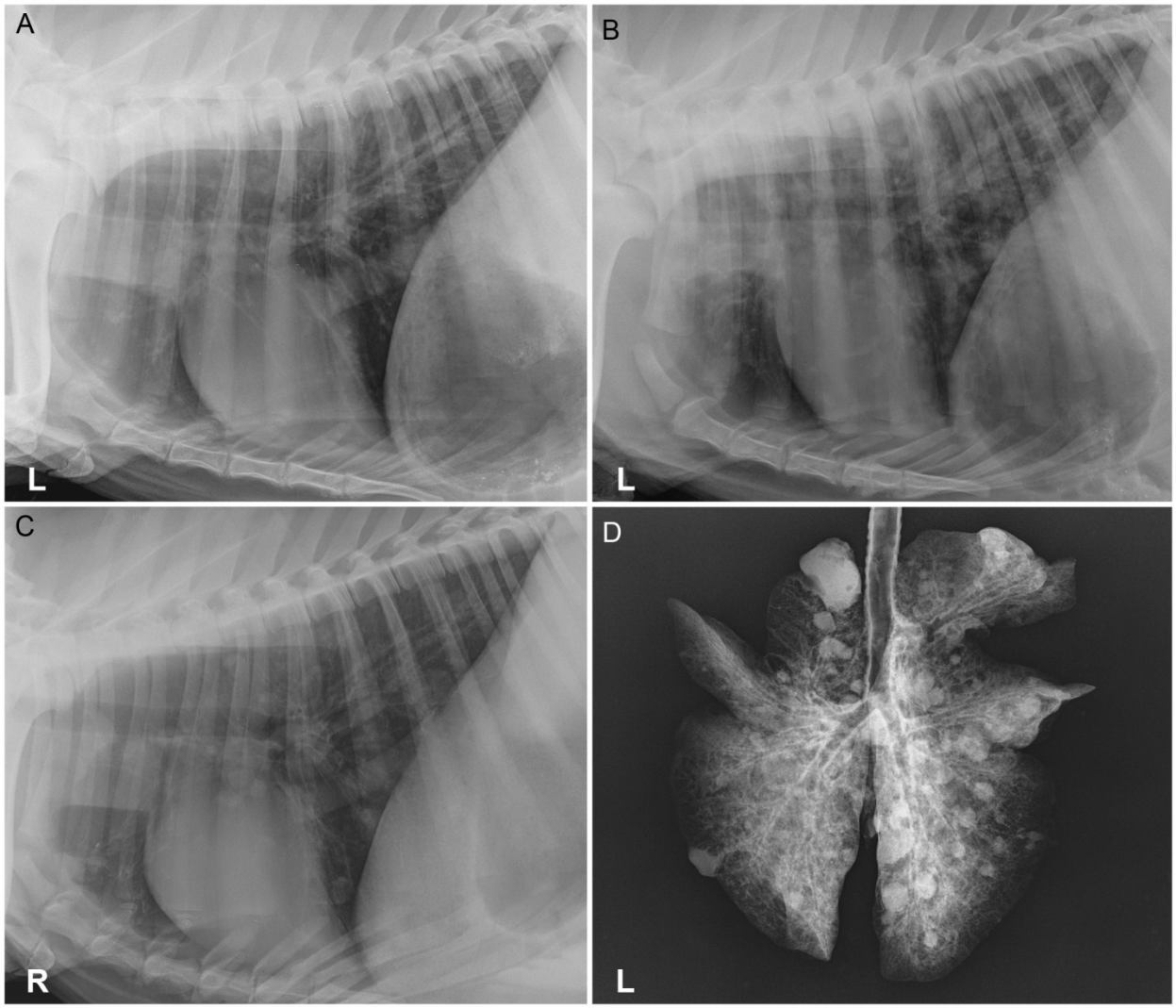

Figure 2.3: Thoracic and extirpated lung radiographs of goats infected with an intratracheal aerosol of *B. pseudomallei*. A) Goat 15, Day 7, left lateral thorax with a mild bronchointerstitial pattern restricted to the caudal lung lobes. B) Goat 16, Day 7, left lateral thorax with moderate to severe bronchointerstitial infiltrates with numerous ill-defined variably sized nodules in all lung lobes with the caudal lung lobes more affected. C) Goat 22, Day 21, right lateral thorax with small nodules seen in all lung lobes and mild-moderate bronchointerstitial infiltrates diffusely. D) Goat 22, Day 21, extirpated lungs, numerous nodules in all lung lobes, which are much more distinct than in the *in vivo* radiographs.

measurements of the nodules ranged from 4 mm to 27 mm in diameter. When compared to *in vivo* thoracic radiographs (Figure 2.3C), radiographs of the extirpated lungs (Figure 2.3D) were similar, but always showed a greater number of and more clearly defined pulmonary nodules.

2.4f) Gross Pathology

Mild focal consolidation in the cranioventral and caudodorsal lung fields was noted in the two goats euthanized on Day 2. Retropharyngeal and tracheobronchial lymph nodes were moderately enlarged. No other lesions were noted in any other organs.

By Day 7 pulmonary abscessation was present in each of the three goats examined. Abscesses were typically 5 mm or smaller and had a creamy to tan center containing variable amounts of thick purulent material. The parenchyma immediately surrounding the abscess was consolidated, appearing as a rim of dark red tissue around the tan center. This configuration was most easily observed in abscesses just beneath the visceral pleura (Figure 2.4A). There was individual variation in the absolute number (range 30 to 90) and distribution of abscesses between right and left lungs, but more numerous and larger abscesses were consistently found in the caudal lung lobes. Variable retropharyngeal, tracheobronchial, and/or mediastinal lymph node enlargement was observed in all three goats on Day 7, which was typically secondary to lymphoid hyperplasia, but was associated with abscessation of the mediastinal lymph nodes in one goat. A 1 mm abscess was observed in the adrenal gland of another goat after formalin fixation. Gross lesions were not observed in any other organs.

On Day 14, pulmonary abscesses remained consistent in appearance and distribution, but varied widely in number (range 12 to 80). The abscesses were larger than at Day 7, most were 5 – 10 mm in diameter, with isolated nodules as large as 20 mm. Retropharyngeal, tracheobronchial, and mediastinal lymphadenomegaly was also consistent with the Day 7 findings. Abscessation of the mediastinal lymph node was seen in one goat, which appeared as multiple small (1 – 2 mm) caseous nodules. This was typical for most lymph node abscesses, which were small in size and multiple in most cases.

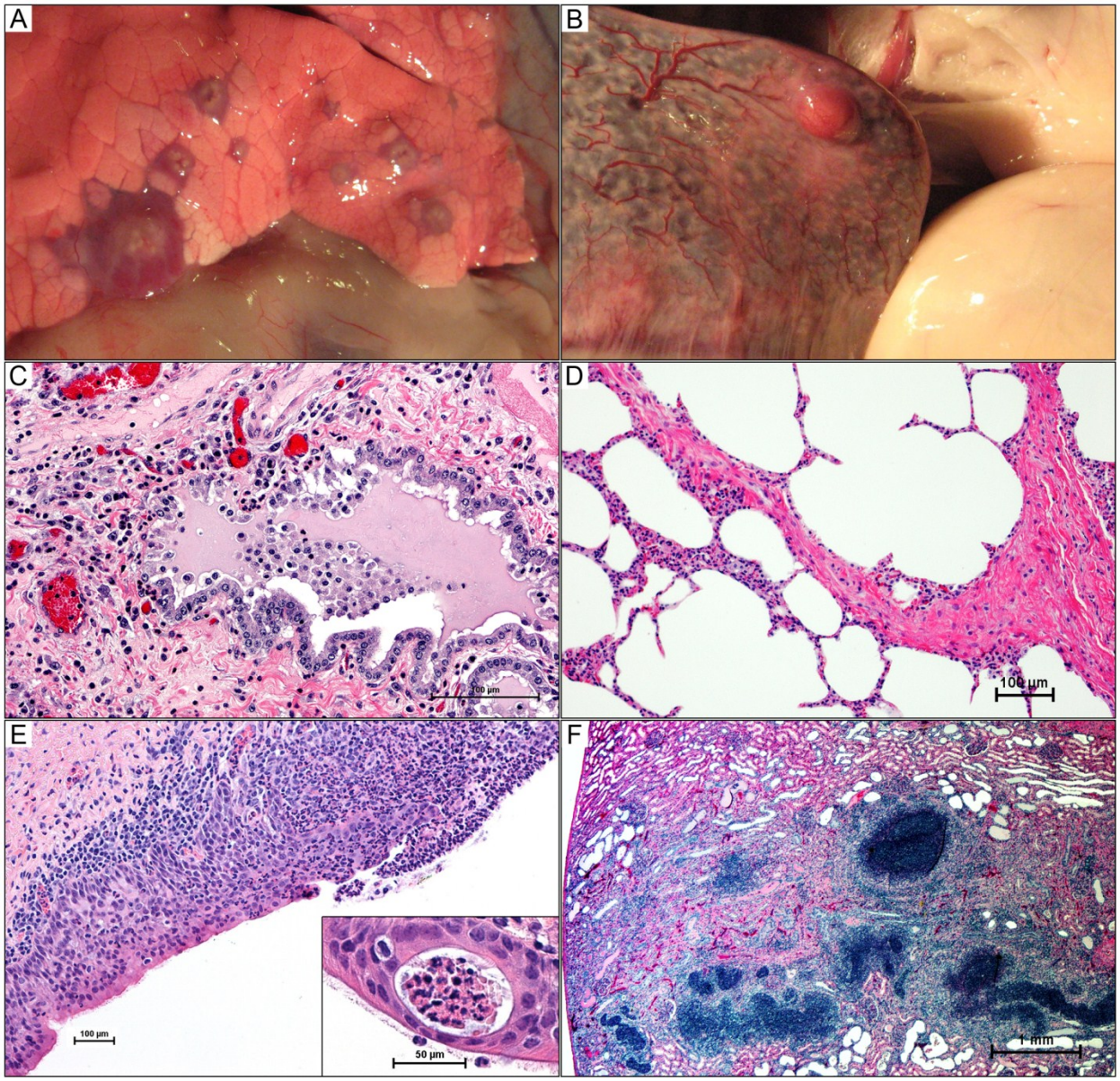

Figure 2.4. Gross and histologic lesions of caprine melioidosis. A) Lung, multiple discrete subpleural targetoid pyogranulomas with tan purulent centers and consolidated hyperemic rims; B) a splenic subcapsular pyogranuloma bulging over the splenic surface with regional capsulitis and injected vessels; C) bronchiolar epithelium showing apical surface deciliation, segmental necrosis of the pseudostratified ciliated epithelium, neutrophil transcytosis, and luminal aggregates of neutrophils suspended in inflammatory edema; D) pulmonary septal thickening secondary to neutrophil infiltration; E) sever necrosuppurative/ulcerative tracheitis with neutrophil transcytosis and a mucosal abscess (inset); F) linear renal pyogranuloma extending from the cortex down into the medulla obliterating large areas of renal parenchyma. 2.4C – F hematoxylin and eosin staining.

Extrapulmonary abscessation was evident in all goats euthanized on Day 14. Splenic abscesses (Figure 2.4B) were seen in all three goats, with one to six abscesses ranging from 2 – 8 mm in diameter. On cut surface, abscesses were white and appeared solid when small (2 – 3 mm), but were caseopurulent in appearance when larger (> 5 mm). In one goat, a renal abscess was observed beneath the capsule, but not adhered to it. On cut section, the abscess had a linear profile, extending from the cortex down into the medulla, but it did not grossly appear to reach the renal pelvis. The central core of the abscess was white to cream color, which was surrounded by renal parenchyma that was discolored tan.

Goat 16, assigned to the group of three scheduled for euthanasia on Day 21, was the most severely affected animal in the study. On Day 16, the goat was acutely moribund and was humanely euthanized. Approximately 50% of the lungs were affected with numerous abscesses of varying sizes up to 25 mm in diameter. Abscesses were seen in the retropharyngeal and tracheobronchial lymph nodes, spleen, and kidneys, which were similar in appearance to the abscesses seen at earlier time points. The left adrenal gland was enlarged and edematous, and when cut longitudinally, it was found to contain a solitary abscess that lacked a distinctive structure with no apparent capsule.

Two goats survived with minimal clinically-evident disease until euthanasia on Day 21. Pulmonary abscessation with widely disseminated disease was evident in both goats at necropsy. Lung abscesses range from 20 to 45 in number and 10 to 20 mm in diameter. One very large abscess in the cranial lung lobe was adhered the visceral pleura to the rib cage. In both goats, abscesses were seen in the tracheobronchial lymph nodes, spleen (grossly enlarged by >10 abscesses in one goat), and kidney. Abscesses in the mediastinal lymph nodes and one small (2

mm) hepatic abscess were also observed, which were similar in appearance to the small splenic abscesses. Organ involvement for individual goats is summarized in Table 2.2.

2.4g) Bacterial Organ Burden

The bacterial organ burden for all goats is summarized in Table 2.2. All grossly evident abscesses (or representative abscesses in the case of multiple abscesses within one organ) cultured positive for *B. pseudomallei*. Positive growth was generally restricted to areas of gross lesions, with the exception of Day 2 samples and tracheobronchial lymph nodes, both of which often cultured positive in the absence of gross lesions. Multiple organs from goat 16 also cultured positive, with relatively high levels of growth, which appeared to be associated with fulminant septic shock.

Only goat 16 was positive for growth of *B. pseudomallei* from neat urine with 4.4×10^4 CFU/mL.

2.4h) Histopathology

Lung. Early bronchiolar lesions at 2 days PI comprised any combination of the following changes: apical surface deciliation; single cell necrosis of the lining pseudostratified ciliated epithelium; neutrophil transcytosis; and luminal aggregates of mucopurulent to fibrinopurulent exudate (Figure 2.4C). Neutrophilic exudate extended into corresponding respiratory ducts and occasionally into adjacent alveoli with expansion of alveolar septa by variable numbers of macrophages and fewer lymphocytes (Figure 2.4D). Perivascular edema and small capillary thrombosis with fibrin exudation was also evident in the most affected areas or animals. Some sections presented with prominent lymphangectasia and lymphoid hyperplasia, with many lymphoid follicles having well developed germinal centers.

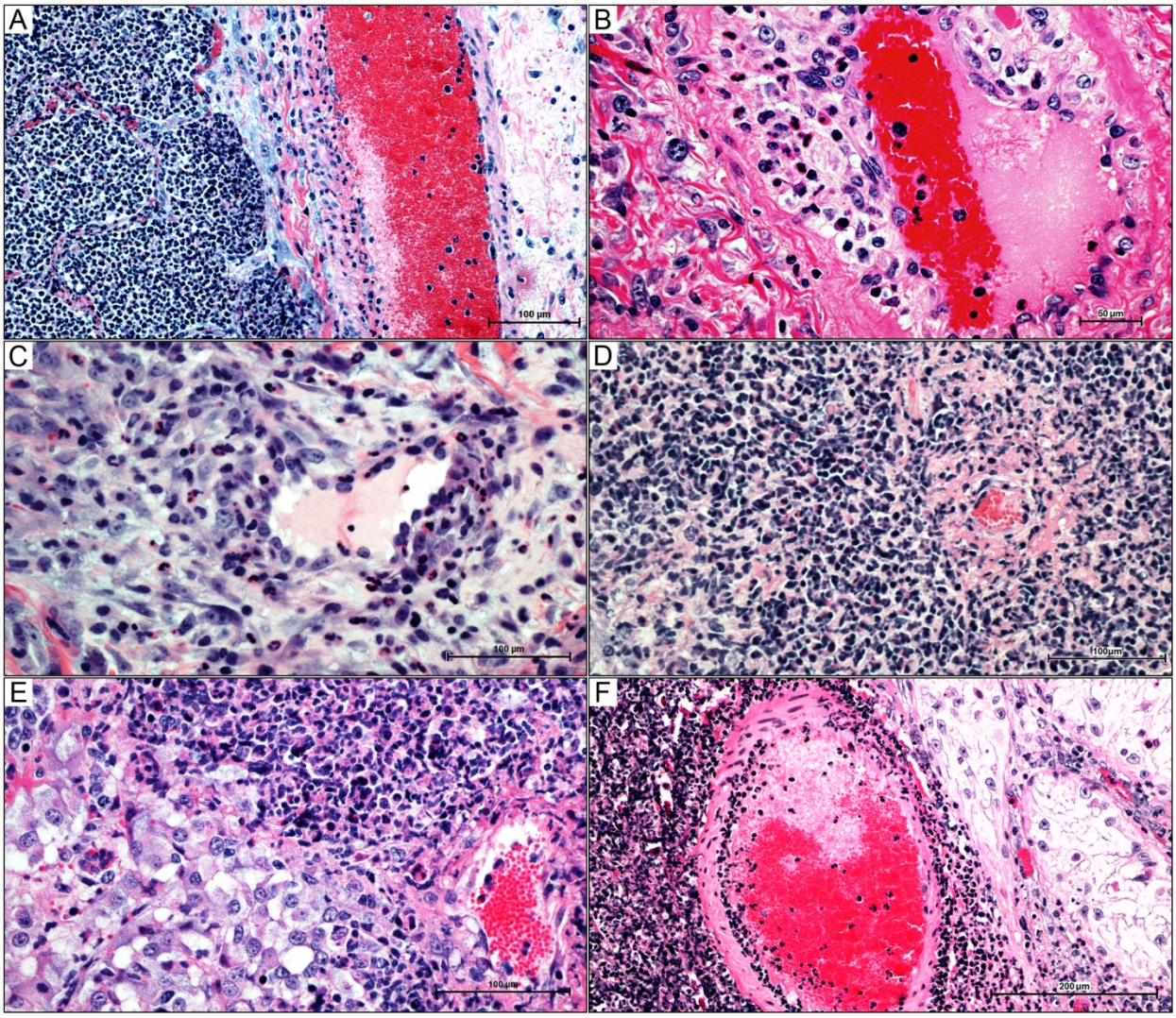

Figure 2.5. Caprine melioidosis vasculitides in multiple organs. A) pulmonary pyogranuloma encroaching on large interlobular vein, resulting in a segmental leukocytoclastic vasculitis – unaffected endothelium is present on the contralateral side of the vessel; B) pulmonary vasculitis in a medium-sized artery demonstrating a clear separation of the tunica intima from the tunica media by neutrophils and macrophages with segmental intimal proliferation; C) splenic capsular vasculitis with perivascular neutrophilic infiltrate ; D) vasocentric renal pyogranuloma showing neutrophil infiltration into the subintima and adventitia with endothelial proliferation and fibrinoid necrosis of a small renal vessel E) focal vasculitis and early pyogranuloma formation at the corticomedullary junction of the adrenal gland; F) leukocytoclastic arteritis in the testicle with an adjacent area of testicular degeneration showing diminished stratification of spermatogenic cells and interstitial suppurative orchitis. 2.5A – F, hematoxylin and eosin staining.

By Day 7 well-formed abscesses were cuffed by mantles of macrophages (including epithelioid histiocytes) and lymphocytes, which in turn were ensheathed by fibrous connective tissue capsules of varying thickness with a significant histiocytic component. Many of the intervening alveolar spaces were lined by plump cuboidal epithelium indicative of proliferation of type 2 pneumocytes. Interlobular septa were also thickened by edema and inflammatory infiltrates, extending to the pleura. Pleural surfaces were variably expanded by pleocellular exudate, subpleural edema, fibroplasia, and lymphangectasia and lymphangitis. A prominent segmental leukocytoclastic vasculitis with perivascular cuffs of lymphocytes and histiocytes (Figure 2.5A and 2.5B) primarily effecting veins was also present.

Lesions were less exudative and more chronic on Day 14, with multiple coalescing pyogranulomas and suppurative to fibrinopurulent bronchopneumonia. Goat 16, which developed terminal disease on Day 16, showed marked pulmonary pathology. Vascular thromboses with perivascular hemorrhages were evident throughout the lungs, which showed fibrin exudation into parenchyma and interlobular septa, prominent peribronchiolar lymphoid hyperplasia, and chronic pleuritis with lymphangectasia and lymphangitis. Lesions on Day 21 were more of chronic bronchointerstitial pneumonia with septal fibrosis, multifocal coalescing pyogranulomas, multifocal atelectasis, and pleural fibrosis.

Trachea. Variable tracheitis, with mucous gland hyperplasia, neutrophil transcytosis, and mucosal abscesses (Figure 2.4E), were seen in all goats at all time points. The severity of the suppurative process appeared most intense on Day 2, and then seemed to decrease in severity with time, having only a mild, focal tracheitis present on Day 21. The exception to this pattern was goat 16 that developed terminal disease, which had severe necrosuppurative tracheitis and

marked neutrophil transmigration, edema, and mucosal abscesses, but lacked hyperplasia of mucous glands.

Tracheobronchial Lymph Node (TBLN). Mild to severe lymphoid hyperplasia, with occasional edema, was seen in all goats that were euthanized on Days 2 to 14. Focal to multifocal pyogranulomas were present in all goats euthanized on Days 16 and 21, and all appeared chronic (with thick fibrous capsules) on histopathology.

Mandibular and Retropharyngeal Lymph Nodes. Mild to severe lymphoid hyperplasia was seen at all time points in all goats. The severity of hyperplasia was less in the mandibular lymph node compared to the retropharyngeal lymph node in all cases. Changes in these lymph nodes did not appear to correlate with the severity of hyperplasia in the TBLNs.

Liver. Histologically, the predominant lesion – regardless of time point – was a minimal to mild random necrosuppurative hepatitis, which was seen in six goats. Hepatic lipidosis was also noted in six goats, but was not associated with significant inflammation as the two lesions were only seen together in two goats.

Spleen. The only findings on Days 2 and 7 were congestion, with mild lymphoid hyperplasia additionally noted in one goat. By Day 14, capsular changes were present (not necessarily directly associated with abscesses), which varied slightly between individual animals, but included suppurative capsulitis with mesothelial cell hyperplasia and hypertrophy, lymphangectasia and lymphangitis, vasculitis, edema, and fibrosis (Figure 2.5C). Vasculitis within the parenchyma was also seen in goat 16. Abscesses had the typical appearance of the pyogranulomas that were observed in the lungs, regardless of their association with the capsule or parenchyma. Pyogranulomas were typically larger by Day 21, but did not appear as active as the lesions on Day 14. Mild lymphoid hyperplasia was also noted throughout the parenchyma.

Kidney. The apparent histologic age of renal lesions was less associated with the duration of infection than pulmonary or splenic lesions. No significant findings were seen in goats on Day 2, nor in one of three goats on Days 7 and 14. 'Early' lesions were seen in two of three goats on Day 7 and one of three goats on Days 14 and 21. These lesions ranged from focally hypercellular glomeruli only to glomeruli showing multifocal to global thickening due to hypertrophy of the parietal layer of Bowman's capsule, mesangial thickening, hyperemia, minimal to mild infiltration of neutrophils, as well as variable tubulointerstitial nephritis and mineralization. The remaining three goats (one each on Days 14, 16, and 21) all had 'late' lesions of characteristic linear abscesses extending from the cortex down into the medulla, but not involving the inner medulla or pelvis. Microscopically, the lesions appeared to be centered on the glomeruli and then spread to the tubules, culminating in tubulointerstitial nephritis with protein casts. The lesions were pyogranulomas with a core of degenerate neutrophils surrounded by a mantle of lymphocytes, histiocytes, and epithelioid macrophages (Figure 2.4F). Vasculitis involving both arteries and veins was seen in association with the renal lesions in two goats. Affected vessels were expanded and segmentally to circumferentially obliterated by neutrophil infiltration into the subintima and adventitia with endothelial proliferation, and/or fibrinoid necrosis (Figure 2.5D).

Adrenal gland. Acute suppuration/abscessation (without fibrosis/capsule) was noted in two goats on Days 7 and 16. This spread by Day 7 was the first evidence of dissemination beyond the pulmonary system and directly draining lymph nodes. Lesions were seen at the corticomedullary junction and appeared to originate from blood vessels, which had evidence of local vasculitis (Figure 2.5E)

Table 2.2: Summary of organ burdens.

Goat	**13**	**18**	**14**	**19**	**27**	**15**	**20**	**26**	**16**	**21**	**22**
Days PI	*2*	*2*	*7*	*7*	*7*	*14*	*14*	*14*	*16*	*21*	*21*
Lung	6.5×10^4	2.4×10^3	Abs	Abs	Abs	Abs	Abs	Abs	Abs	Abs	Abs
Spleen	NG	NG	NG	NG	NG	Abs	Abs	Abs	Abs	Abs	Abs
Liver	NG	NG	NG	NG	NG	NG	NG	NG	1.2×10^4	NG	Abs
Kidney	NG	NG	NG	NG	NG	Abs	NG	NG	Abs	Abs	Abs
RPLN	NG	NG	NG	NG	NG	NG	NG	NG	1.7×10^4	NG	NG
TBLN	8.5×10^2	NG	6.0×10^2	2.5×10^2	NG	6.5×10^2	NG	NG	Abs	Abs	Abs
MedLN*	NE	NE	NE	NE	Abs	NE	NE	Abs	NE	Abs	NE
MesLN	NG	NG	NG	NG	NG	NG	NG	NG	8.0×10^2	NG	NG
Urine (CFU/mL)	NG	NG	NG	NG	NG	NG	NG	NG	4.4×10^4	NG	NG

RPLN: retropharyngeal lymph node, TBLN: tracheobronchial lymph node, MedLN: mediastinal lymph node, MesLN: mesenteric lymph node, NG: no growth of *B. pseudomallei*, NE: not examined, Abs: grossly abscessed, *MedLN only collected if abscessed

Mesenteric Lymph Node. The mesenteric lymph node was typically observed to be edematous with mild to moderate lymphoid hyperplasia regardless of time point.

Testis. Multifocal suppurative orchitis was noted only in goat 16, which was associated with vasculitis (both arteritis and phlebitis), hemorrhage, testicular degeneration, azospermia, and giant spermatids (Figure 2.5F).

2.5) Discussion

Melioidosis has a diverse clinical presentation in humans. This may be affected by individual characteristics of the infecting strain, dose, and route of infection, which is rarely known in naturally occurring cases. These unknowns make it very difficult to study the pathogenesis of melioidosis despite the large number of individual case reports and several retrospective and prospective studies. Basic pathogenesis research has been largely restricted to murine models, but additional models are necessary for a more complete understanding of the pathogenesis of infection with *B. pseudomallei*, which has a very broad host range. Additionally, new animal model systems are also important for the development and testing of novel therapeutics and vaccines.

Patients with melioidosis typically present with protean signs. Since there is no "typical" presentation, the subdivision of cases is highly variable in the human medical literature, with some papers grouping patients by duration of signs (acute vs. chronic), the presence or absence of septic shock/bacteremia, or the primary organ system involved. Pneumonia, with (76%) or without (45%) septic shock, accounts for the largest percentage of clinical presentations of naturally occurring human melioidosis [8]. In pulmonary cases, signs that are typically present include fever (50 – 100% of patients) [76,173] and leukocytosis (70% of patients) [175].

In our goat model, the only consistent clinical sign of infection was fever. The degree of pyrexia appeared to parallel the increase in granulocyte count over the first seven to nine days, with peaks on Day 1 and around Day 7. After Day 9, temperatures tended to decrease, but this decrease was not necessarily paralleled in the granulocyte count (see Figures 2.1 and 2.2). The consistency of these alterations is expected early in infection when the dominant variables of route of infection, dose, and strain are all controlled for by the nature of experimental infection. As disease progresses over time, individual host factors may become more important and account for the greater variability seen.

Other clinical signs included cough and oculonasal discharge. Nasal swabs, including those from goats with nasal discharge, were typically culture negative for *B. pseudomallei* except in the case of goat 16, where increasingly heavy discharge and growth of *B. pseudomallei* was associated with the progression towards terminal disease. Light positive growth seen on Day 1 was interpreted as residual bacteria from intratracheal infection rather than dissemination or expectorated pus. This low rate of nasal shedding suggests that horizontal spread among animals via nasal secretions would be unlikely or insignificant. This appears similar to human disease where there are very few reports of person-to-person transmission [14] even though 68% of patients with evidence of pulmonary involvement are positive on sputum culture [176].

Hematogenous dissemination, the only mechanism consistent with the observed pattern of multiorgan involvement, was not directly confirmed, likely because of the transient nature of bacteremia. Positive blood cultures were only seen in goat 16, which was associated with the progression to terminal disease. The bacteremia observed in goats appears similar to the bacteremia seen in human patients, in which only 50% of patients are culture positive, and of the culture positive patients the median bacteremia is 1.1 CFU/mL [185]. Urine collected at

postmortem examination was only positive in one of the four goats with renal abscessation. The goat with a positive urine culture had terminal disease at the time of collection, which could suggest that urinary shedding is a late event and/or indicative of a more severe or advanced expansion of renal abscesses into the pelvis. This could be consistent with the finding that the presence of a positive urine culture is a negative prognostic indicator in human melioidosis patients [144]. Counts ranging from $10^4 – 10^6$ CFU/mL were also seen in a small number of goats used in the dose determination portion of this study (data not shown), which is comparable to counts seen in human patients [144,185].

Thoracic radiographic findings in human melioidosis are highly variable and the incidence of specific findings also varies considerably among retrospective imaging studies of melioidosis. Radiographic signs in acutely presenting cases often have local, patchy, alveolar infiltrates, or disseminated nodular lesions, which are suggestive of metastatic (hematogenous) spread [175,178-181]. Acute cases tend to have much more rapid deterioration and death [183]. The findings in subacute to chronic presentations are more variable, with some studies reporting the absence of a predominant lesion [178], while others report findings very similar to what is seen in acute cases [175]. The upper lobes appear to be more affected in melioidosis, especially in subacute/chronic cases [174,175,178,180-182]. The predominant radiographic changes seen in the goats after aerosol exposure were bronchointerstitial infiltrates indicative of airway inflammation; pulmonary nodules were often initially identified in the caudal lungs and subsequently spreading to all lung lobes. This distribution was as expected with aerosol delivery (with or without secondary septicemic seeding of the lungs), but radiographically could have the same appearance as abscesses formed from the hematogenous spread of bacteria. Therefore, it is possible that the nodular appearance in human cases could be the result of inhalational and/or

septicemic disease. Despite aerosol delivery of bacteria and acute fever, there was not rapid progression of disease radiographically or clinically. This is partially a function of dose as was seen in dose determination studies, where a delivered dose of 10^7 CFU produced fulminant pulmonary disease, necessitating euthanasia 48 h PI. Additionally, the use of healthy animals, without any predisposing risk factors for melioidosis, could have limited the development of rapidly progressive disease.

Organ involvement in melioidosis can be highly variable in both humans and goats. In humans, the lungs are the most commonly affected organ, but virtually any organ can be affected [8,14]. Data concerning organ distribution of naturally occurring melioidosis lesions seen in goats has been described in abattoir studies from Malaysia [443] and Australia [439,442]. The pattern of organ involvement seen in the goats in these studies [439,442,443], previous experimental infections [470,471], and our current model is generally similar to what is seen in human disease, with the spleen, lung, kidney, and liver being commonly affected.

One notable difference in organ involvement and lesion severity observed in goats in this study compared to mice and humans was the rarity of gross hepatic abscesses and only mild severity of microscopic changes within the liver. Previous reports of both natural and experimental melioidosis in goats have had variable findings in regards to liver lesions, with some reporting no lesions [439,470] and others finding hepatic lesions to be quite common [105,442,443,471]. This finding may be a result of strain variation, individual host/population factors, or the duration of infection. Hepatic (and splenic) abscesses are much more common in Thai patients than Australian patients [6,8,175,407], which again may relate to strain variation and duration of illness.

In the current study, the lungs were the primary site of infection. Dissemination to extrapulmonary tissues appeared to be predominantly a function of time, with greater numbers of organs involved at the later time points. Dissemination to sites other than lymph nodes draining the pulmonary system was not grossly evident before Day 14 with the exception of one small adrenal abscess seen in one goat on Day 7. The histologic evidence suggests that the timing of dissemination was a result of the size and extent of the pulmonary pyogranuloma formation. After aerosol delivery, mucopurulent bronchopneumonia rapidly developed and soon progressed to a more severe fibrinopurulent to bronchointerstitial pneumonia with pyogranuloma formation evident radiographically, grossly, and histologically by Day 7. Within the lungs, the lesions spread along interlobular septa and subpleural stroma inducing a local leukocytoclastic vasculitis where the pyogranulomas encroached upon and eventually invaded the vessel (Figure 2.4D). The vasculitis appeared to peak in severity around Day 7. The rise in temperatures around Day 7 as well as the gross appearance of the majority of the extrapulmonary lesions on Day 14 or later supports the central role of the vasculitis in hematogenous dissemination of *B. pseudomallei*.

Vasculitis also appears to be the central pathologic step in the establishment and progression of pyogranuloma development in extrapulmonary organs. Vasculitis within an adrenal vessel was noted in association with abscess formation and invasion of the parenchyma. Splenic lesions in the capsule and parenchyma were also seen in association with vasculitis. The predominance of capsular pyogranulomas appears to correlate with the capsulitis observed histologically. The renal lesions suggest that the point of entry for *B. pseudomallei* is the glomerulus, with the earliest detectable lesion being hypercellular glomeruli with neutrophil infiltration. The lesions then suppurate and extend into tubules creating tubulointerstitial disease and ultimately result in the formation of pyogranulomas. The testicle was the only organ with

lesions that had evidence of hemorrhage in association with the vasculitis. The significance of this finding is unclear at this time since a testicular lesion was only seen in one goat, but lends support to vasculitis being a central event in pathogenesis.

To the authors' knowledge, vasculitis has not previously been reported as a feature of human disease or in murine models. It has been reported in goats in association with central nervous system lesions [439]. There are a limited number of papers with detailed histopathologic descriptions of human disease [420-425], none of which report vasculitis. The majority of these reports are based on acute fatal cases with lesions that are more suppurative in nature. Zones of hemorrhage, particularly surrounding pulmonary lesions are frequently reported in acute human cases [420-424], but have not been reported to be associated with vasculitis. A more recent paper, which also included a number of surgical biopsy samples from chronic cases in addition to acute fatal cases, specifically noted that vasculitis was not found, and only reported hemorrhage being variably present in biopsy samples [425]. The lack of vascular lesions in human cases may be a species difference or could reflect the skewed distribution of histologic samples in human cases.

Goats are generally believed to have a natural tendency towards more chronic disease and granulomatous type lesions in melioidosis [420], though disease ranging from acutely fatal to apparently self-curing lesions has been observed [445]. While there are certain notable differences in caprine and human melioidosis, the ability of goats to survive the acute stages of melioidosis, contain *B. pseudomallei* to more chronic lesions, and even potentially eliminate the organism, makes them a particularly interesting animal model of melioidosis. We have shown that melioidosis can be readily induced in goats following aerosol exposure. As expected, the extent of organ involvement seen was more variable in goats than in murine models, but was

comparable to human disease and is likely to be a feature of any model with a truly heterogeneous outbred population. However, it appeared that organ involvement may become more consistent at later time points with more chronic disease in our caprine model.

The larger body size of goats allows for human-relevant clinical monitoring as well as longer-term serial evaluation of disease progression and therapy. We believe the caprine model will be a useful animal model for further investigation of the molecular pathogenesis and host response in melioidosis, as well as evaluation of preventative and therapeutic interventions.

CHAPTER 3: ROUTE OF INFECTION AND DISEASE PATHOGENESIS IN SUBACUTE TO CHRONIC CAPRINE MELIOIDOSIS

3.1) Summary

Melioidosis is a severe suppurative infection caused by *Burkholderia pseudomallei*. Disease is endemic to Southeast Asia and Northern Australasia, but is also of global interest because of its designation as a Category B select agent. Experimental infection models have repeatedly shown inhalational infection to be the most lethal form of disease, which is also supported by epidemiological studies of natural disease. Natural infection can occur by percutaneous inoculation, inhalation, or ingestion, but the relative importance of each route is unknown. Few studies have examined the pathogenesis of percutaneous infection despite its presumptive importance in natural disease. Caprine models are very useful in the study of natural infection in people because goats are naturally affected by melioidosis with similar epizootiology/epidemiology within the endemic range and develop similar pathologic lesions. Percutaneous inoculation with 10^4 CFU of *B. pseudomallei* produced disease in all animals with rapid dissemination to the lungs, spleen, and kidneys. Initial fever was brief, but temperatures did not return to pre-infection levels until Day 18 in association with a dramatic increase in peripheral lymphocytes and the transition to chronic disease. Lesion distribution and appearance was very similar to caprine aerosol infection and human disease. The similarities seen despite very different routes of infection suggest that host factors may be more important to disease presentation and progression. The subacute to chronic nature of melioidosis in goats makes them amenable for the additional modeling of risk factors and acute disease, which is very important in human disease.

3.2) Introduction

Burkholderia pseudomallei has been recognized as the causative agent of melioidosis for just over a century in Southeast Asia and Northern Australasia [1]. Melioidosis has always been associated with significant morbidity and mortality but was considered rare for the 80 years following its discovery [15]. In the early 1990s it was recognized as an emerging infectious disease and the subsequent study of melioidosis and *B. pseudomallei* has grown exponentially over the last 20 years. A significant additional driver of research over the past 10 years has been the designation of *B. pseudomallei* as a Category B select agent by the United States Centers for Disease Control and Prevention because of its potential use in bioterrorism. However, the immediate significance of melioidosis as a naturally occurring disease is highlighted by its mortality rate, which has made it the third most common infectious cause of death in Northeast Thailand [57].

The clinical epidemiology, presentations, diagnosis, and treatment of melioidosis have been examined in many prospective and retrospective studies, which have led to a much greater understanding of human disease. However, the study of bacterial virulence factors, disease pathogenesis, and the development of novel preventative and therapeutic measures is fundamentally dependent on animal models of disease. Model development for the study of human disease has been pursued in the mouse [459,462,476,482,483,487], hamster [460,464], rat [461,463], pig [468], goat [492], marmoset [472], rhesus macaque, and African green monkey [473]. The infrastructure and resources of biomedical research facilitates the use of murine models, which have dominated the study of melioidosis with only minor efforts being devoted to other species. However, the impetus for the development of novel models is to provide a comparative approach for the study of melioidosis as well as a much needed second animal

model for the assessment of new therapeutic and preventative measures required prior to entering human clinical trials [387].

The success and utility of these models must be assessed in terms of the goals for the models and their limitations for the study of melioidosis. The majority of murine [462,482,485] and all recent non-human primate (NHP) models [472,473] focus on acute inhalational disease. Much of this was born out of the realm of biodefense, though the importance of natural inhalational infection [84,91,92] is supported to a similar extent as percutaneous infection [52,53,79,81] by epidemiological data.

Partially by design and partially by inherent species susceptibility, many models reside on extremes of the spectrum of clinical disease. Very high infecting doses are needed to produce disease in pigs, outbred mice, or rats, though some of this appears to be a function of route of infection as rats can be infected more easily intratracheally than intraperitoneally [461,463,468,474]. In contrast, hamsters, BALB/c mice, and NHPs are exquisitely sensitive to *B. pseudomallei*, which can be mediated by route of infection in BALB/c mice [482,484] and some NHPs [457,472,473], but not in hamsters [464]. While these extremes in animal models of melioidosis can be useful for specific questions relating to the pathogenesis (mechanism for increased susceptibility following pulmonary exposure in the rat) or assessing therapeutics in NHPs, it greatly limits the ability to model the spectrum of human disease in a single species or add additional layers of complexity such as predisposing risk factors, which are present in 80% of melioidosis patients [8]. Subacute to chronic models in the C57BL/6 mouse [483] and goat [492] models appear to have the greatest potential to model a spectrum of disease, which could be manipulated through dose, bacterial strain, route of infection, or the addition of predisposing risk factors.

The final limitation to consider for the various model species is the natural occurrence of disease in wild or domestic populations. Natural infection in pigs is typically chronic or asymptomatic (discovered only at slaughter) and likely follows oral infection [40,41,439,443,445,450]. Pigs have repeatedly been shown to be very resistant to experimental infection – even with immunosuppression [430,468,469], such that they do not appear to be a well-suited species for the study of melioidosis. Disease in NHPs has been reported as well, but of the species used for experimental models, natural disease (outside of zoological gardens) has only been reported for two rhesus macaques [428]. Both exhibited chronic or reactivated latent disease, with diagnosis 6 months and 10 years after acquisition [494,495].

The goat, as a naturally affected species, has a base of scientific literature describing the presentation and lesions of caprine melioidosis [441-443,445,446,470,471,492], which compares well with human disease in terms of clinical presentation, epizootiology [429], organ distribution, and histopathology [422,425]. While goats more frequently develop chronic disease, acute presentations are possible and therefore goat models are likely amenable to the incorporation of risk factors to increase susceptibility/acute disease.

The goat model provides an opportunity to study the relative importance of the route of infection. Disease and outcome can be compared to natural presentations in both human and goat populations which have greater relevance since goats and humans are similarly exposed to environmental *B. pseudomallei*. The objective of this study was to create a non-fulminant, subacute to chronic percutaneous infection model of caprine melioidosis and compare its pathogenesis to aerosol infection relative to natural disease. Disseminated melioidosis was rapidly produced in all percutaneously infected goats. Disease was generally similar to but more variable than aerosol infection.

3.3) Materials and Methods

3.3a) Bacterial Strain and Culture Methods

An Australian isolate of *B. pseudomallei* (Bp 4176/MSHR 511) [491] was generously provided by Dr. Apichai Tuanyok. Bacteria for infection were grown fresh in Muller-Hinton (MH) broth (M5887 Teknova, Hollister, CA, USA) at 37°C in air with constant shaking at 250 RPM. Bacteria were harvested in mid-log phase growth. Based on the OD600, the culture was diluted in phosphate buffered saline (PBS) to achieve a final concentration of 5×10^4 CFU/mL. A target dose of 1×10^4 CFU in 0.2 mL of PBS was selected based on pilot studies, which investigated a dose range of $8.2 \times 10^2 - 10^6$ (data not shown). The bacterial suspension used for infection was backtitrated in duplicate on MH agar plates incubated at 37°C in air.

In an effort to improve the sensitivity of blood culture from previous experiments [492], a larger volume of blood (8.3 mL) was sterilely collected into 1.7 mL sodium polyanethanol sulfonate (SPS, 0.35% in 0.85% sodium chloride) anticoagulant (BD Vacutainer® 364960, Franklin Lakes, NJ, USA). Anticoagulated blood was then mixed with 190 mL of molten (45°C) MH agar and poured into three 15 cm Petri dishes for quantitative culture [345]. Once solidified, the plate was incubated at 37°C in air. Plates were monitored for seven days before being declared negative. Colonies seen on blood culture plates were subcultured onto selective media (MH agar with 4 mg/L gentamicin [MH-gentamicin]) to verify gentamicin resistance and colony morphology consistent with *B. pseudomallei*. Nasal swabs, organ homogenates, and abscess material were also cultured on MH-gentamicin plates at 37°C in air and read at 96 h, 48 h, and 48 h, respectively.

All experiments using *B. pseudomallei* were performed in a biosafety level-3 facility.

3.3b) Experimental Animals

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and every effort was made to minimize suffering. The protocol was approved by the Animal Care and Use Committee of Colorado State University (approval 11-2414A). Seventeen adult Boer-cross goats were obtained through private sale for use in this experiment. Animals were obtained from herds that had no known history and tested negative for Caprine Arthritis Encephalitis and Johne's Disease (*Mycobacterium avium* subsp. *paratuberculosis*). Caseous lymphadenitis (CL) (*Corynebacterium pseudotuberculosis*) was present in some source flocks and goats with visible lesions were excluded, as were the results from any goats with CL lesions found at necropsy.

Goats weighed 35 to 100 kg. There were fourteen females and three castrated males. Animals were housed in an animal biosafety level-3 (ABSL-3) facility for the duration of the experiment. They had *ad libitum* access to water and were fed a complete pelleted feed twice daily, supplemented with grass hay. Goats were acclimatized to the facility for approximately one week prior to infection. The bacterial suspension was inoculated (Day 0) into the dermis (0.1 mL) and subcutaneous tissue (0.1 mL) overlying the caudolateral aspect of the brachium, for a total target dose of 10^4 colony forming units (CFU).

3.3c) Clinical Monitoring: hematology and clinical microbiology

Beginning on Day -3, the goats' attitude, appetite, and temperature were monitored daily for the duration of the experiment. Normal rectal temperature was defined as 38°C to 40°C. Four pre-infection complete blood counts (CBCs) (HemaTrue Hematology Analyzer, Heska Corporation, Loveland, CO, USA) were performed on each goat to establish baseline

hematologic values. Post-infection (PI) CBCs were performed on Days 1-7, 9, 11, 14, 18, 21, 25, 28, 32, 35, 39, and 42.

Previous blood collection for culture based on a set schedule yielded very few positive results [492]. Therefore, blood cultures collection was targeted for only Days 1-5 and then for any day where the rectal temperature was over 39°C and had increased at least 0.6°C over the temperature from the previous day. The collection site over the jugular vein was aseptically prepared with povidone iodine and ethyl alcohol, and blood was collected into a sterile tube prefilled with SPS anticoagulant.

Nasal swabs of both nostrils were sampled daily for the duration of the experiment with a Dacron® fiber tipped plastic applicator swab (Cat. No. 14-959-90, Fisher HealthCare, Houston, TX, USA) and immediately streaked onto a MH-gentamicin agar plate.

3.3d) Thoracic Radiography

Two-view thoracic radiographs (right lateral and left lateral) were taken under xylazine sedation for all goats prior to infection and on Days 7, 14, 21, 28, 35, and 42. A ventrodorsal view was not included as previous experience showed it to be of limited diagnostic use. Radiographs of the extirpated lungs were also taken at the time of necropsy. Initial studies showed little to no radiographic change in the lungs on Day 2; therefore, this necropsy time point was not aligned with radiography. Radiographs were taken using a MinXRay® 100HF and Agfa® Computed Radiography (CR) 43x35 CR MD 4.0 General Cassettes. During use within the ABSL-3, cassettes were triple sealed in 6 mil polyethelene bags (LAD 8555, Hillas Packaging, Inc., Fort Worth, TX, USA). The cassettes were processed with a CR 85-X digitizer and the images were processed with NX software.

3.3e) Euthanasia, Necropsy, Histology, and Organ Burden

Humane euthanasia of goats with intravenous pentobarbital was planned for Days 2, 7, 14, 21, and 42 PI. Five goats were assigned to the Day 42 time point to ensure there were at least three goats surviving to Day 42. All other time points had three goats assigned. Necropsies were performed on all goats. Based on organ involvement detected in pilot studies, the following tissues were collected into 10% neutral buffered formalin for histology: infection site (skin, subcutis, and underlying muscle), prescapular lymph nodes (PSLN), retropharyngeal lymph node (RPLN), tracheobronchial lymph node (TBLN), mediastinal lymph node (MedLN), lungs, heart, spleen, liver, kidney, adrenal, thyroid, brain, and mesenteric lymph node (MesLN). Samples were routinely embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for microscopic evaluation. Selected tissues were also examined with Brown-Hopps staining when bacteria were visible with hematoxylin and eosin staining.

In organs with macroscopic abscesses, the abscesses were counted or estimated as a percentage of the total organ volume. Representative abscesses were cultured on MH-gentamicin agar to confirm positive growth of *B. pseudomallei*. In the absence of macroscopic abscesses, samples of the following organs were aseptically collected to calculate the organ burden (CFU/g): lung (cranial, middle, and caudal lobes of left and right lungs), spleen, liver, kidney, prescapular lymph node, mediastinal lymph node, and mesenteric lymph node. Tenbroeck homogenizers were used to homogenize 0.5 g of each tissue sample in 2 mL PBS + 20% glycerol (limit of detection 25 CFU/g). Urine was also collected and cultured from each goat at the time of necropsy, and cultured as neat urine and as a urine pellet (4 mL centrifuged at 7,500 x g for 10 minutes). The homogenate or urine (100 μL) was plated in duplicate on MH-gentamicin agar, and the remaining sample was frozen at -80°C. If the colony count was above

the limit of quantitation (>300 colonies/plate), the frozen homogenate was thawed and serial log dilutions were plated in duplicate.

3.3f) Cytokine Measurement

Serum was collected for cytokine analysis prior to infection and on Days 2, 7, 14, 21, and 42 PI. Post-infection sera were filtered using a 0.2 μm syringe filter, cultured for sterility, and frozen at -80°C prior to analysis. Pre-infection sera were centrifuged at 10,000 x g for 10 minutes to remove platelets prior to analysis (filtration eliminated platelets in PI samples). Sera from goats infected in a previous experiment (Chapter 2) by intratracheal aerosol [492] were similarly collected and stored for analysis alongside samples from percutaneously infected goats. Analysis was performed using commercially available enzyme linked immunosorbent assay (ELISA) kits for bovine CCL2 (VS0083B, Kingfisher Biotech, Inc., St. Paul, MN, USA), bovine IFNγ (VS0257B, Kingfisher Biotech, Inc., St. Paul, MN, USA), bovine IL-10 (BV1495, TSZ ELISA, Framingham, MA, USA), and TGF-β1 (MB100B, R&D Systems, Minneapolis, MN, USA). Optimal dilution of sera for CCL2 and IFNγ was determined to be 1:8. All other protocols were followed according to the manufacturer's instructions. Plates were read using a BioTek Synergy 2 multi-mode microplate reader and Gen5 software (BioTek Instruments, Inc., Winooski, VT, USA).

3.3g) Data Analysis

Summary measures of clinical data are reported as means and ranges. All statistical calculations were performed with SAS Version 9.3 (SAS Institute, Cary, NC). The association between temperature, granulocyte count, and lymphocyte count was analyzed by the Corr procedure using Pearson correlation. Temperature, granulocyte count, lymphocyte count, and

serum cytokine concentration data were analyzed using the mixed procedure for repeated measures ANOVA with an autocorrelation model (selected by AICC). Logarithmic (base 10) transformation was used for cytokine data to normalize data with outliers remaining after transformation excluded from analysis, which was only necessary for BpG48 in the analysis of IFNγ. Temperature, granulocyte count, and lymphocyte count data were analyzed for the effect of day, and cytokine data were analyzed for the effect of day, route, and route x day interaction. The Day 42 results were excluded for any analysis including a route effect. Results were considered significant with a p-value < 0.05.

3.4) Results

3.4a) Bacterial dose

Infection was performed on three separate days. The backtitration of the bacterial suspension ranged from 7.7×10^3 to 9.9×10^3 CFU. The delivered dose to individual goats is reported in Table 3.1.

3.4b) Clinical Monitoring

The mean rectal temperature for all goats prior to infection was 38.6°C. Day 2 was the only day of the experiment where the mean temperature (40.1°C) was in the febrile range. Seventy-six percent (13/17) of goats were febrile on Day 2. Of the four goats that were not febrile on Day 2, two never became febrile and the others developed fevers on Days 5 or 8. While not meeting the definition of fever (>40°C), mean temperatures were above the mean pre-infection temperature by 0.7 to 1.2°C from Days 3 through 17, which were all significantly

Table 3.1: Goats infected with *B. pseudomallei* and status at euthanasia.

Goat No.	Sex	Dose (CFU)	Day PI of Necropsy	Status at Euthanasia/Death
BpG43	F	9.9×10^3	2	Febrile acute disease
BpG51	F	9.2×10^3	2	Febrile acute disease
BpG52	F	9.2×10^3	2	Febrile acute disease
BpG45	F	9.9×10^3	7	Subclinical subacute disease
BpG46	F	9.9×10^3	7	Subclinical subacute disease
BpG47	F	9.2×10^3	7	Febrile
BpG42	F	9.2×10^3	9	Died, hypothermic, purulent nasal discharge, severe acute progressive disease
BpG50	F	9.9×10^3	14	Subclinical subacute disease
BpG44	F	9.9×10^3	14	Febrile subacute disease
BpG49	F	9.9×10^3	21	Subclinical chronic disease
BpG48	F	9.2×10^3	21	Subclinical chronic disease
BpG54	F	9.2×10^3	21	Febrile active disease
BpG55	F	8.5×10^3	42	Subclinical chronic disease
BpG56	MC	7.7×10^3	42	Subclinical chronic disease
BpG57	MC	8.5×10^3	42	Subclinical chronic active disease
BpG58	MC	8.5×10^3	42	Subclinical chronic active disease
BpG59	F	8.5×10^3	42	Subclinical chronic active disease

higher than Day 0. Day 18 was the first day that the mean temperature was not significantly different from Day 0, with the mean temperatures on Days 1 – 17 all additionally being significantly higher than Day 18. The mean temperature was not different from Day 0 for 17 of the remaining 24 days, indicating an overall return to pre-infection levels. However, two goats (BpG54 and 59) notably maintained temperatures well above pre-infection levels and often in the febrile range for the duration of the experiment (21 and 42 days, respectively). Temperatures were quite variable within and amongst goats over the first two and a half weeks of the experiment. The variability of temperatures of individual goats as well as the mean temperatures over the duration of the experiment is shown in Figure 3.1.

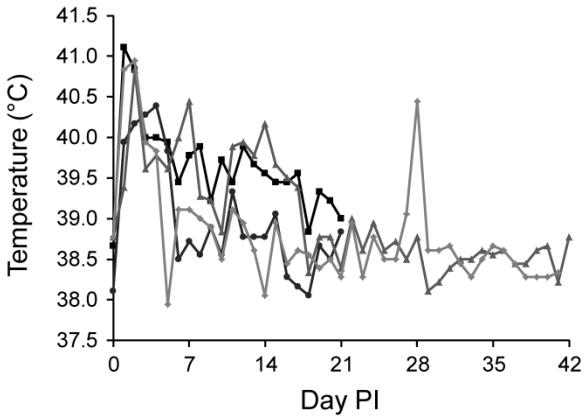

Figure 3.1: Temperatures from selected goats. Peaks in temperature are commonly seen on Days 1 and 2 before returning to pre-infection levels after Day 18 and the predominance of chronic disease. The peak in BpG57 on Day 28 highlights the chronic active nature of disease at later time points. BpG48 (■), BpG49 (●), BpG56 (▲), BpG57 (♦).

Despite high fevers (up to 41.4°C) lethargy or decreased appetite was rarely observed. Two goats were mild to moderately lethargic on Day 2 in association with high fevers. Moderate lethargy and inappetance were first noted on Day 8 in BpG42, which rapidly deteriorated to severe lethargy, obtundation, and death on Day 9 (before euthanasia could be performed). This goat was the thinnest of all the animals and never developed a fever.

The most frequent clinical sign recognized was swelling at the site of infection and enlargement of the ipsilateral prescapular lymph node. The subcutaneous swelling organized into abscesses that ruptured in 80% (8/10) of goats that survived through Day 14 (no abscess ruptured in any goat prior to Day 14). Lameness was only noted in one goat in association with an injection site abscess that was quite large, but did not rupture. Cough was intermittently noted in two goats (BpG54 and 59). Significant nasal discharge was rarely observed, with one goat developing purulent discharge three days prior to death (BpG42) and another (BpG54) briefly developing hemorrhagic discharge on Day 16.

3.4c) Hematology

Pre-infection hematology was generally unremarkable in regards to red cell parameters, and no remarkable changes were observed during the course of infection. White cell parameters were more variable during the pre-infection sampling, which were attributed to a cortisol response from handling during the acclimatization period. The mean pre-infection white blood cell counts for all goats over all independent samples was as follows: total white blood cells 11.9 x 10^3 cells/μL (range 4.9 – 26.3 x 10^3 cells/μL, reference range 4 – 13 x 10^3 cells/μL); lymphocytes 4.8 x 10^3 cells/μL (range 2.4 – 7.7 x 10^3 cells/μL, reference range 2 – 9 x 10^3 cells/μL); monocytes 0.8 x 10^3 cells/μL (range 0.2 – 1.6 x 10^3 cells/μL, reference range 0 – 1 x 10^3 cells/μL); and granulocytes 6.4 x 10^3 cells/μL (range 1.6 – 21.2 x 10^3 cells/μL, reference range 1.2 – 7.2 x 10^3 cells/μL). While some leukocyte counts were elevated at individual time points, only one goat (BpG42) maintained mildly elevated granulocyte count for all samples. All other goats and leukocyte parameters measured within the reference range for at least one pre-infection time point.

The only cell types with a notable post-infection response were granulocytes and lymphocytes (see Figure 3.2). On Day 1 the mean granulocyte count significantly increased to 9.0 x 10^3 cells/μL and again on Day 2 to 10.2 x 10^3 cells/μL. The mean granulocyte counts remained significantly elevated above Day 0 through Day 35 and above the high end of the reference range through the end of the study. This peak on Day 2 was followed by a more gradual but higher peak that was significantly higher than the Day 5 trough for Days 6 to 32, returning to pre-infection levels on Days 39 and 42. The granulocyte count was moderately positively correlated with temperature ($r = 0.41$, $p < 0.0001$).

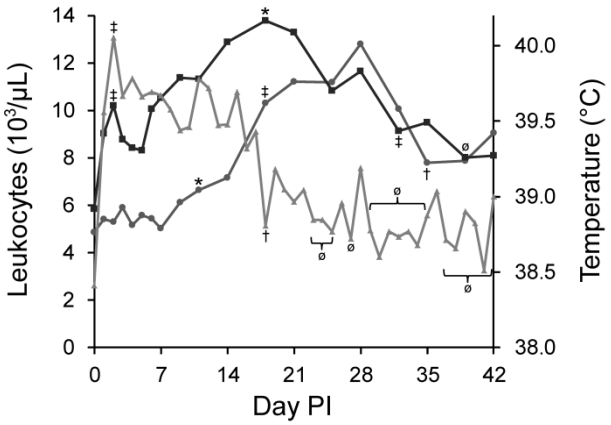

Figure 3.2: Mean granulocyte count, lymphocyte count, and temperature. Granulocytes (■) showed an initial peak on Day 2 and then on Day 18 before a significant decrease on Day 32 and a return to pre-infection levels on Day 39. Lymphocytes (●) first increase over pre-infection levels on Day 11, with a significant increase out of the normal range on Day 18, which is associated with a significant decrease in temperature (▲) to pre-infection levels. Lymphocytes significantly decrease to pre-infection levels on Day 32. (‡) different than previous measure and pre-infection, (†) different than previous measure but no different than pre-infection, (*) different than pre-infection, (ø) not different than previous measure or pre-infection, significance $p < 0.05$.

The mean lymphocyte count was generally unremarkable for the first 14 days of the experiment. Significant increases above Day 0 but within the normal range were observed on Days 11 and 14. The greatest significant increase lymphocyte count and out of the normal range was seen on Day 18, which peaked on Day 28 with 12.8 x 10^3 cells/µL before returning to pre-infection levels and the normal range on Day 35. The lymphocyte count showed a weak, but significant negative correlation with temperature ($r = -0.16$, $p = 0.027$)

3.4d) Clinical Microbiology

Nasal swabs were positive for *B. pseudomallei* in 35% (6/17) goats over the course of the entire study. Of the six goats that cultured positive only three were noted to have grossly evident nasal discharge, which was aligned with the timing of the certain positive cultures from two goats. Four of the six goats to have positive nasal cultures were in the six week group, but

otherwise there was no pattern of timing or distribution of cultures, which ranged from only a single positive culture, to intermittent shedding, or positive culture for nearly all of the latter three weeks of the study. Thick nasal discharge with heavy growth of *B. pseudomallei* was associated with terminal disease in goat BpG42 but not in any other goat. Growth from the nasal swabs was typically light to moderate.

Quantitative blood culture was negative for *B. pseudomallei* for all goats at all time points.

3.4e) Thoracic Radiography

All pre-infection radiographs for all goats were within normal limits except for BpG51, which appeared to have chronic changes from a previous episode of pneumonia (no clinical or hematologic finding supported active disease prior to infection). Pulmonary disease was radiographically evident in 64% (9/14) goats by Day 7. Changes ranged from a mild local bronchointerstitial pattern in the dorsal aspect of the caudal lung lobes to moderate diffuse bronchointerstitial infiltrates with or without nodular opacities (4-6 mm), suggesting small abscesses or pyogranulomas associated with pneumonia (Figure 3.3A). Extirpated lung radiographs of the goats euthanized on Day 7 showed the same bronchointerstitial infiltrates but revealed more numerous small nodules throughout the lung lobes (which was typical for all extirpated radiographs compared to *in vivo* imaging). BpG42 has the worst changes with interstitial coalescing to alveolar infiltrates and 1-2 cm nodules in the dorsal aspect of the caudal lung lobes, which progressed to diffuse nodular interstitial to alveolar infiltrates in all lung lobes at the time of death on Day 9. Radiographic evidence of pneumonia was present in 90% (9/10)

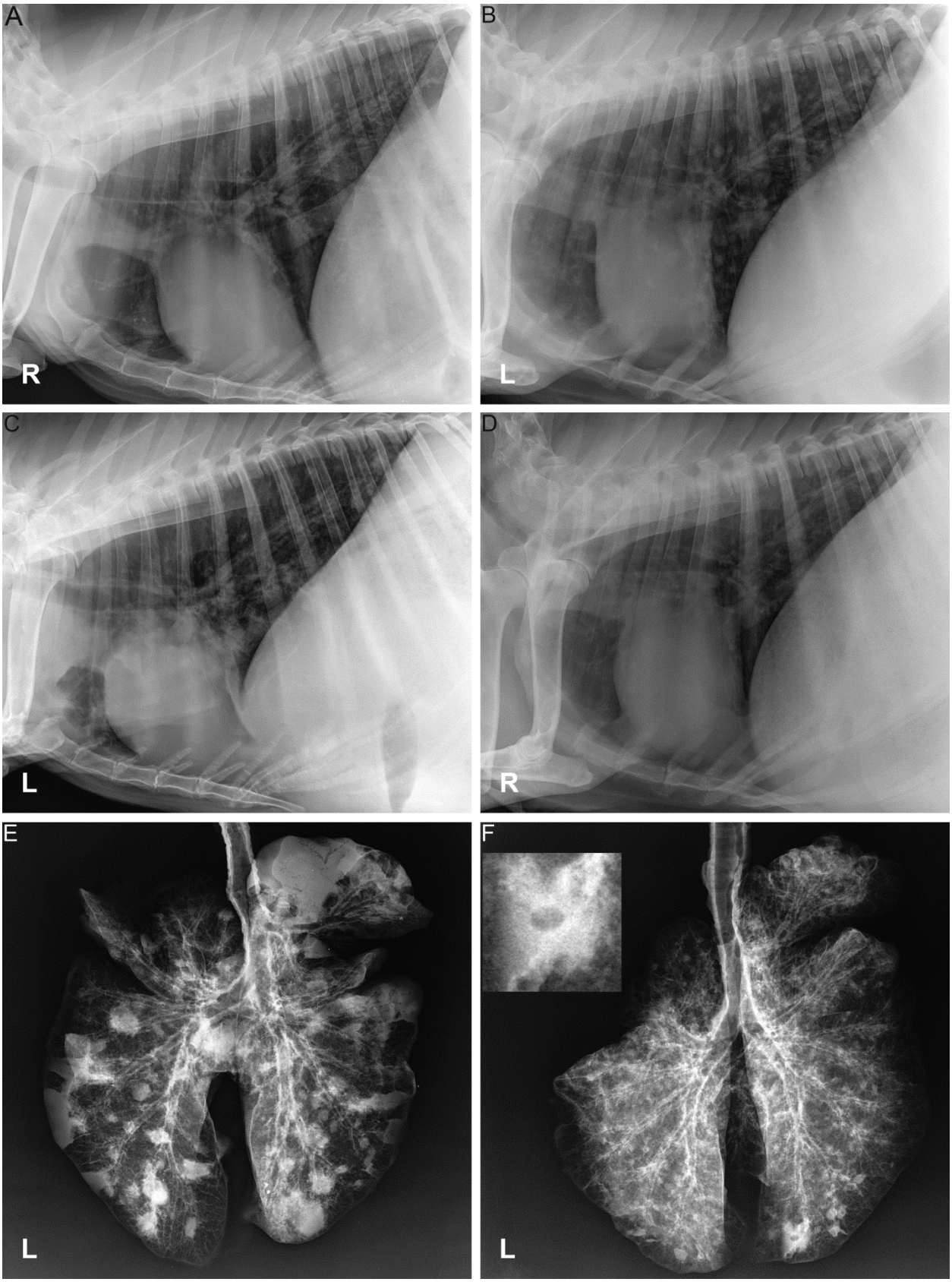
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Figure 3.3: Thoracic and extirpated lung radiographs of goats infected percutaneously with *B. pseudomallei*. A) BpG47, Day 7, right lateral thorax, moderate bronchointerstitial infiltrates and small ill-defined nodular infiltrates in the dorsocaudal and cranioventral lung lobes. B) BpG59, Day 14, left lateral thorax, moderate-severe bronchointerstitial infiltrates and numerous nodules (5-11 mm) throughout the pulmonary parenchyma. C) BpG54, Day 21, left lateral thorax, moderate diffuse bronchointerstitial infiltrates, patchy alveolar infiltrates in the mid-caudal lung lobes, bronchoalveolar infiltrates in cranioventral lung lobes, and numerous nodular opacities (6-16 mm) throughout the pulmonary parenchyma. D) BpG59, Day 42, progression of the bronchointerstitial infiltrates with several poorly defined opacities/alveolar infiltrates within the lungs, though nodules are less defined than on Day 14. E) BpG54, Day 21, extirpated lungs, numerous nodules and several focal areas of consolidation throughout the parenchyma with a dense bronchial pattern diffusely. F) BpG59, Day 42, extirpated lungs, severe diffuse bronchointerstitial pattern with a large 2 cm cavitated nodule (inset) in the right caudal lung lobe with several other nodules in the remaining lungs.

goats by Day 14. The changes were generally progressive, with increasing patchy (more caudodorsally) to diffuse bronchointerstitial infiltrates that follow the caudal bronchi seen in goats with radiographic changes. Bronchointerstitial changes range from mild to severe, with occasional peribronchial cuffing and alveolar infiltrates. Multiple ill-defined, variably sized (5-11 mm) soft tissue opaque nodules are present in 67% (6/9) goats, which are very numerous in individual goats (Figure 3.3B). All eight goats had radiographic evidence of disease on Day 21, with half of the goats having static lesions. Progression was typified by more widespread and/or worsening bronchointerstitial and alveolar infiltrates, more numerous and larger nodules (3-20 mm) (Figures 3.3C and E). Nodules were observed in 75% (6/8) goats. The remaining five goats had unchanged lesions on Days 28 and 35 with the exception of BpG56, where nodular lesions were less distinct than Day 21. By Day 42 the nodular lesions in BpG55 and 56 were distinctly smaller (Figure 3.3D). Worsening bronchointerstitial and alveolar infiltrates were seen in two goats, while no evidence of progression was seen in the remaining two goats. Nodular

lesions were visible in all extirpated lungs, with a 20 mm cavitated lesion observed in BpG59 (Figure 3.3E).

3.4f) Gross Pathology

The only acute lesion observed on Day 2 associated with the experimental infection was edema in the subcutaneous tissues at the injection site. Lesions associated with chronic pneumonia were observed in BpG51, corresponding to the reported radiographic findings, along with an abscessed mediastinal LN. A focal 2 cm tan lesion was also noted in the liver of BpG52, which appeared to be a chronic abscess.

By Day 7 abscesses were present at the site of infection in two of three goats and in the prescapular lymph node ipsilateral to the site of infection in all three goats. Skin and prescapular LN abscesses appeared as 2 – 4 mm discrete white/cream colored lesions with caseopurulent centers within the dermis and/or subcutis and ipsilateral LN. Abscesses were present in the lungs of all three goats, the spleen of one goat, and the kidney of another goat. The mediastinal LN was greatly enlarged, but an abscess was not noted until the LN was sectioned after formalin fixation. In the lungs the center of the abscesses were surrounded by a dark red rim of consolidated parenchyma. The kidney lesions appeared as small (3 – 4 mm diameter) nodules protruding above the surface, which when cut revealed a cream colored, linear, and variably-sized abscesses originating in the cortex and expanding down towards the medulla, with the adjacent parenchyma discolored tan.

BpG42 was the only goat to die from the experimental infection. While a small abscess was observed at the site of infection, there was minimal edema of surrounding tissues and no abscessation of the ipsilateral prescapular LN. Gross lesions were consistent with fulminant disease, with edematous connective tissue throughout the body with noticeable subpleural

edema, fibrinous pleural adhesions, and serosanguinous hydrothorax. The lungs were consolidated, firm, and filled with small, extensive, multifocal ("miliary") abscesses (Figure 3.4A). The only other organ with a visible abscess was the kidney, which was very small (1 mm surface diameter) but had the characteristic linear structure when cut. Lymphadenomegaly was observed in the mediastinal and mesenteric LNs. Very little abdominal fat was present.

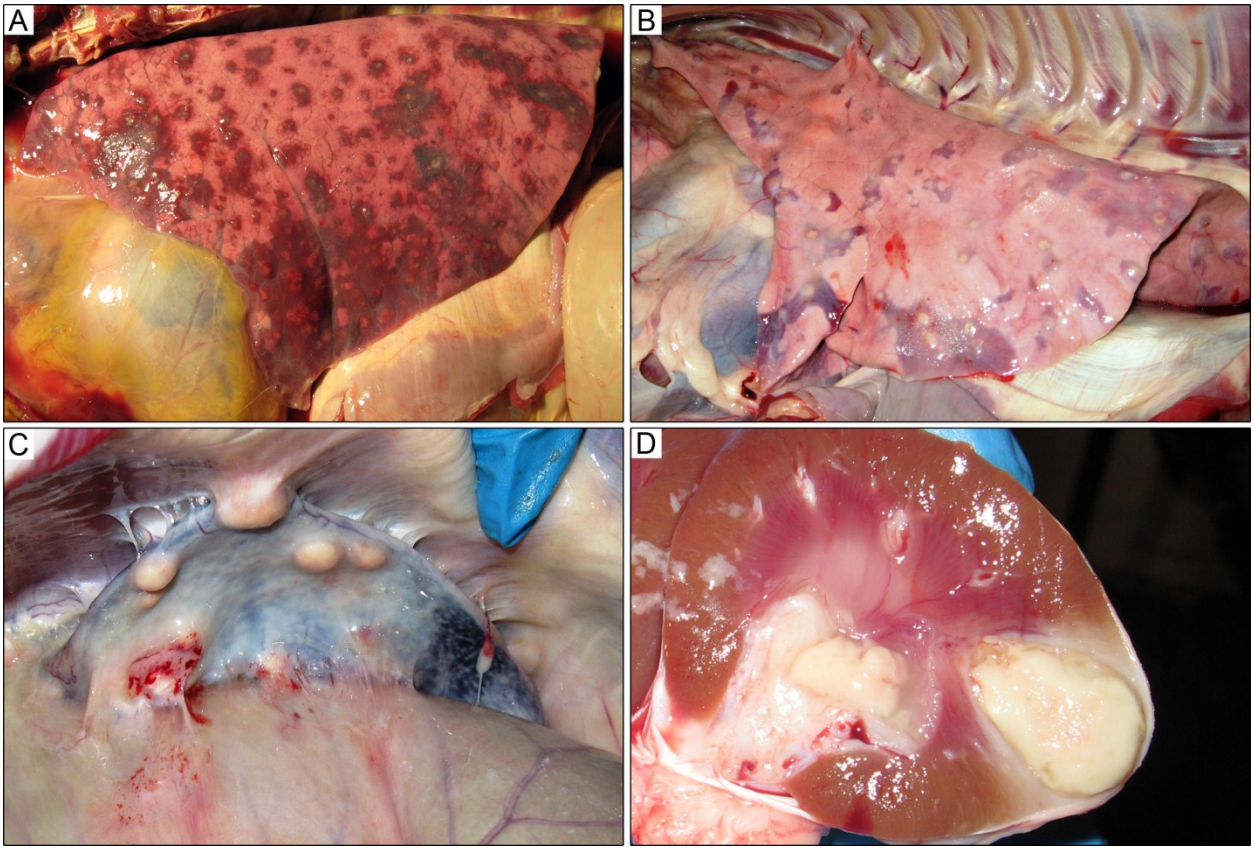

Figure 3.4. Gross lesions of caprine melioidosis. A) Lung, numerous, multifocal, discrete to coalescing abscesses/pyogranulomas associated with extensive consolidation and hemorrhage; B) Lung, typical subpleural targetoid pyogranulomas with tan purulent centers and consolidated hyperemic rims that are rarely associated with fibrous pleural adhesions; C) Spleen, multiple, large pyogranulomas with adhesions of the splenic capsule to the peritoneum; D) Kidney, multifocally extensive and coalescing pyogranulomas spanning from cortex to medulla.

Lesions at Day 14 and beyond were variable amongst goats, but affected similar organs as seen on Day 7. Additional lesions observed included pulmonary adhesions (Figure 3.4B), abscesses extending down into the muscle underlying the injection site in two goats, a mammary abscess, and tracheobronchial LN abscessation. Retropharyngeal LN enlargement was noted in two goats, with one goat noted to have abscessation when cut after formalin fixation. The nasal turbinates from BpG59 were filled with mucopurulent exudate. A single liver abscess was noted in one goat (BpG48), which appeared as a 2 mm cream colored nodule. The sublumbar lymph nodes were grossly enlarged, discolored black by draining hemorrhage, and contained small abscesses in one goat (BpG58). BpG50 was markedly less affected than other goats, with grossly visible abscessation at the injection site. A few very small abscesses were also found in the lungs, but only after formalin fixation.

Abscesses were typically more numerous and larger at later time points, particularly for the prescapular lymph node, spleen, and kidneys (Figures 3.4C and D). Splenomegaly was often present by Day 42 with numerous (30 – 40) and large (15 mm) pyogranulomas effacing nearly half of the organ and adhering the spleen to the peritoneum/body wall. Pulmonary abscesses were typically small (~5 mm), though individual goats did have lesions up to 20 mm in diameter. Lung lesions were concentrated in the caudal lung lobes, which was especially true for the larger lesions. The cavitary lesion seen radiographically in BpG59 corresponded with a larger abscess. On cut surface the cavity consisted of an empty necrotic center encased by an irregular wall that was 1-2 mm thick. After formalin fixation several smaller abscesses in this goat were also noted to have very small (1 mm diameter) cavities. Organ involvement for individual goats is summarized in Table 3.2.

Table 3.2: Summary of organ burdens.

Goat No.	Day	Injection Site	PSLN	RPLN	Lung	MedLN	Liver	Spleen	Kidney	MesLN	Urine
BpG43	2	NG	2.5×10^5	NG	NG	NG	NG	NG	NG	NG	NG
BpG51	2	NG	3.6×10^5	NG	25	NG	NG	NG	NG	NG	NG
BpG52	2	3.0×10^3	2.4×10^4	NG	50	NG	NG	25	NG	NG	NG
BpG53	2	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
BpG45	7	2.3×10^2	Abs	NG	Abs	NG	NG	NG	NG	NG	NG
BpG46	7	Abs	Abs	NG	Abs	NG	NG	NG	Abs	NG	NG
BpG47	7	Abs	Abs	NG	Abs	1.0×10^2	NG	Abs	NG	NG	NG
BpG42	9	Abs	6.3×10^3	1.3×10^3	Abs	1.7×10^3	3.0×10^4	2.8×10^5	Abs	1.8×10^2	3.0×10^5
BpG44	14	Abs	3.8×10^2	NG	Abs	25	NG	NG	Abs	NG	NG
BpG50	14	Abs	NG	NG	50	NG	NG	5.5×10^2	NG	NG	NG
BpG48	21	Abs	Abs	NG	Abs	Abs	Abs	Abs	NG	NG	NG
BpG49	21	Abs	Abs	NG	Abs	NG	NG	NG	NG	NG	NG
BpG54	21	Abs	Abs	NG	Abs	Abs	NG	Abs	Abs	NG	10
BpG55	41	Abs	Abs	NG	Abs	75	NG	Abs	Abs	NG	NG
BpG56	42	Abs	Abs	NG	Abs	Abs	NG	Abs	Abs	25	5.5
BpG57	41	Abs	Abs	NG	Abs	NG	NG	Abs	NG	NG	NG
BpG58	42	Abs*	Abs	25	Abs	NG	NG	Abs	Abs	NG	1.0×10^3
BpG59	42	Abs	Abs	Abs	Abs	Abs	NG	Abs	Abs	NG	5.9×10^2

PSLN: prescapular lymph node, RPLN: retropharyngeal lymph node, MedLN: mediastinal lymph node, MesLN: mesenteric lymph node, NG: no growth of *B. pseudomallei*, Abs: grossly abscessed, *Culture negative abscess.

3.4g) Bacterial Organ Burden

The bacterial organ burden for all goats is summarized in Table 3.2. Positive cultures were most common from the lungs and ipsilateral prescapular LN (94%), injection site (88%), spleen (65%), and kidney and mediastinal LN (47%).

B. pseudomallei was isolated from all sampled abscesses, with the notable exception of the abscess at the site of infection in BpG58. Positive growth was generally restricted to areas of gross lesions with the exceptions of lymph nodes, which were often positive with no gross changes or only enlargement. BpG50 cultured positive from the spleen in the absence of gross lesions. Multiple organs from BpG42 also cultured positive, with relatively high levels of growth, which appeared to be associated with fatal fulminant septic shock.

Urine culture was positive in 5 of 17 goats, with one positive culture coming from the pellet only.

3.4h) Histopathology

Skin/infection site: *Acute lesions/Day 2*: Lymphedema with ectatic lymphatics were prominent within the panniculus, which occasionally showed fibrin deposition as well as microscopic hemorrhages. Inflammatory infiltrates of intact and degenerate neutrophils were observed occasionally in parallel lines of perivascular infiltrates, in the deep dermis, panniculus, and interstitial spaces of skeletal muscle. Inflammatory cells permeated adjacent adenexa, cuffing blood vessels and terminal nerves (Figure 3.5A). In addition to perivascular edema, vascular changes included endothelial hypertrophy and hyperplasia, neutrophil margination, hemorrhage, fibrinocellular thrombi in arterioles, and variably severe fibrinoid degeneration with leukocytoclastic vasculitis. *Subacute lesions/Day 7*: The changes were similar to previous lesions

with the progression to multifocal to coalescing pyogranulomas in the superficial and deep dermis and muscle, necrosis of adnexal structures and arterioles, clefting of the overlying epidermis (Figure 3.5A), and granulation tissue formation in the deep dermis, panniculus, and perimysium. Vasculitis was less prominent or absent. *Subacute to chronic lesions/Days 14 – 42*: Lesions progressed to full thickness necrosis (occasionally associated with infarction) of the overlying epidermis with an ulcer bed of mixed bacteria, neutrophils, and granulation tissue. Interstitial inflammation became more lymphocytic, variably extending to the subjacent muscle where fibroblastic interstitial thickening isolated individual hyalinized skeletal muscle fibers. Older pyogranulomas occasionally exhibited multifocal central areas of mineralization. In cases where the infection site abscess/pyogranuloma drained, cicatricle dermal fibrosis was observed. Prescapular LN: *Ipsilateral to Infection Site*: Occasional macrophages in subcapsular sinus were observed to contain intracytoplasmic bacteria. Lesions appeared to begin with a variably severe suppurative capsulitis with a prominent vasculitis that was occasionally associated with hemorrhage. The vasculitis appeared most intense around Day 2 and was not observed after Day 7. Multifocal discrete to coalescing pyogranulomas formed first in the subcapsular area and extended into the nodal cortex (Figure 3.5B). Inflammation tracked along trabeculae (trabeculitis) to deeper seated vessels where more vasculitis/fibrinoid inflammation was seen with subsequent pyogranuloma development in the hilar area. The pyogranulomas had the same general structure as seen in the skin, but at later time points, macrophages and multinucleate cells surrounding the liquefied center showed phagocytic activity with encroachment of the outer histiocytic layer, suggesting early regression of lesions. Later in disease, the LN capsule often became very thickened (up to 10x normal) by granulation tissue and inflammatory exudates,

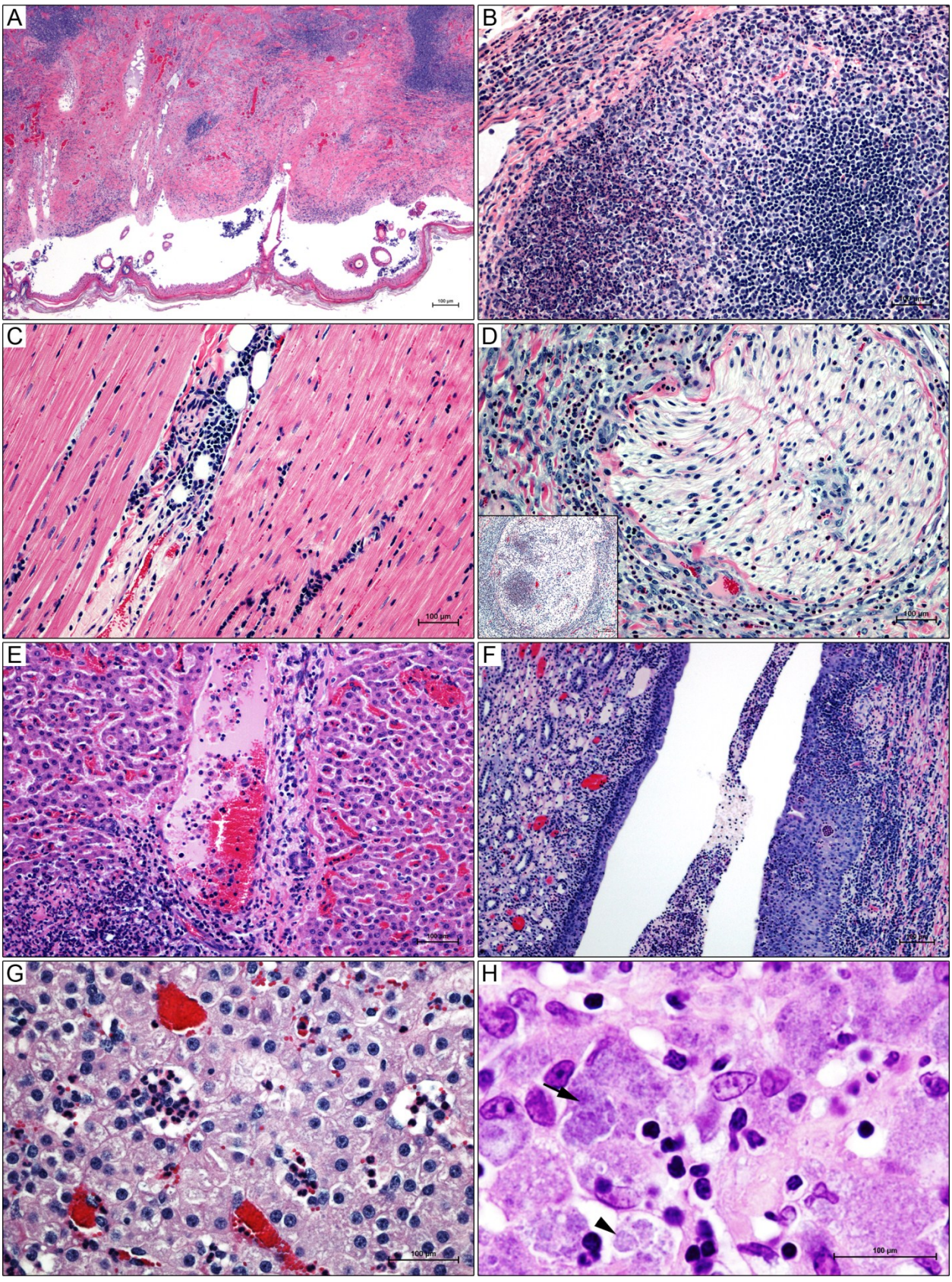
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Figure 3.5: Extrapulmonary histologic lesions of percutaneous caprine melioidosis. A) Skin, multifocally extensive neutrophilic infiltrates expand the deep dermis surrounding vessels and adnexa with sub-epidermal clefting; B) Prescapular LN, capsulitis and early subcapsular abscess involving the subjacent lymphoid follicle ; C) Heart, lymphoplasmacytic interstitial myocarditis infiltrating along tissue planes; D) Spleen, neuritis with suppurative inflammation surrounding and minimally infiltrating a small nerve and forming neural microabscesses in a larger nerve(inset); E) Liver, vasocentric portal microabscess obliterating the lower pole of a portal vein; F) Kidney, suppurative pyelonephritis with degenerate neutrophils filling the lumen, infiltrating the pelvic lining forming a mucosal abscess, and invading the suburothelial stroma; G) Adrenal, microabscess with lytic necrosis are scattered within the zonae fasciculata and reticularis; and H) Mesenteric LN, phagocytically active macrophages with abundant intrahistiocytic (arrow) and extracellular (arrowhead) coccobacilli. Hematoxylin and eosin staining.

which matured into fibrous connective tissue including hypertrophic capsular vessels. Lymphoid hyperplasia was marked in the non-affected (non-abscessed) cortex, and in rare instances lymphocytes were intermixed with moderately large numbers of megakaryocytes – indicative of extramedullary hematopoiesis. Lesions in the ipsilateral node were seen in all goats.

Contralateral to Infection Site: No suppurative inflammation was observed, but non-specific changes of mild to moderate edema, hyperplasia, and sinus histiocytosis was often present.

Trachea: As early as Day 2 lymphoplasmacytic tracheitis was evident with the tracheal submucosa expanded by a band of lymphoplasmacytes with or without multifocal areas of deciliation. Occasional vessels contained increased numbers of neutrophils, which infiltrated the submucosa and insinuated between mucosal cells to the lumen with small numbers of macrophages and lymphocytes (Figure 3.6A). The finding on Day 7 ranged from histologically normal to static lymphoplasmacytic tracheitis to progressive erosive tracheitis with the formation of mucosal abscesses. These lesions were most severe in BpG42 with the submucosa and connective tissue surrounding the trachea dispersed by a large amount of edema fluid and few

inflammatory cells. A neutrophilic component to the tracheitis was observed in 4 of 5 goats on Days 14 and 21, which ranged from transcytosis (most commonly) to deciliation, mucosal abscess formation, and apoptosis and necrosis of the pseudostratified columnar. On Day 42, only minimal to mild lymphocytic to lymphoplasmacytic tracheitis was observed. Overall tracheal lesions were very common, being found in 88% (15/17) of goats.

Lung: Initial mild to moderate changes included edema, atelectasis, and ectasia of interlobular vessels with perivascular neutrophil infiltrates spilling over into alveolar spaces along with small clusters of macrophages. More advanced changes seen on Day 2 included terminal airways and mainstem bronchi filled with suppurative aggregates that infiltrate adjacent alveoli through necrotic epithelium (Figure 3.6B). Severely affected lobules were largely collapsed with a mixed inflammatory infiltrate. Occasionally deciliated bronchiolar epithelium showed surface colonization by coccoid to coccobacillus shaped bacteria. The spectrum of changes seen on Day 7 were continuous with the more severe changes seen on Day 2. Surrounding alveolar spaces were filled with fibrinocellular exudate and scattered alveoli were lined by type 2 pneumocytes. Interlobular septa and connected pleura were expanded by edema, inflammatory infiltrates (neutrophils, lymphocytes, plasmacytes, and macrophages) occasionally associated with granulation tissue, and mature fibrous connective tissue. Vasocentric abscesses surrounded necrotic vessels, which were associated with microscopic hemorrhage and thrombosis (Figures 3.6C, D, and E). Various stages of early abscessation through mature pyogranulomas were observed. Leukocytoclastic vasculitis was randomly distributed throughout affected lobules, which was evident in medium sized arteries with intimal proliferation with scattered mitotic figures (Figure 3.6F). By Day 14, lesions were still active with necrosuppurative bronchiolitis to fibrinopurulent bronchopneumonia and vasculitis. However, components of chronic

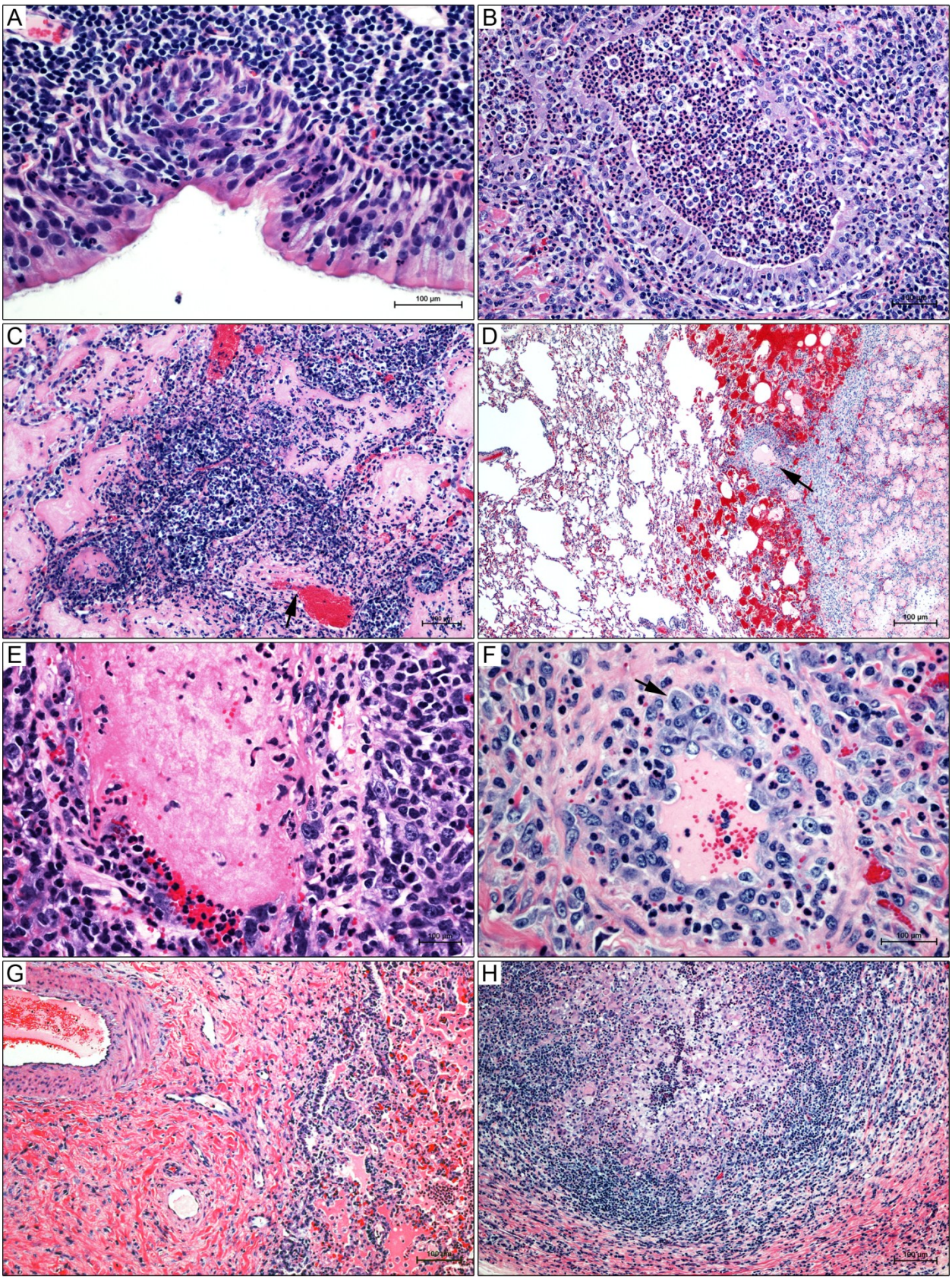
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Figure 3.6: Respiratory lesions of percutaneous caprine melioidosis.
A) Suppurative tracheitis showing neutrophil transcytosis and submucosal expansion by a thick band of lymphoplasmacytes; B) Suppurative bronchiolitis with focal necrosis of the bronchiolar wall and luminal aggregates of neutrophils; C) Early vasocentric abscess formation, neutrophils exiting vessel (arrow) and filling perivascular alveolar and interalveolar spaces; D) Microscopic pulmonary hemorrhage centered on interlobular vessel (arrow), hemorrhage to the left of interlobular septum and edema to the right; E) A fibrinocellular thrombus occludes the lumen of a small vein; F) Small arteriole, typical lesion of leukocytoclastic vasculitis with neutrophil infiltration into the wall and medial hyperplasia, evidenced by a mitotic figure (arrow); G) Chronic active bronchointerstitial pneumonia showing septal fibrosis alongside alveolar edema, suppurative inflammation, and pneumocytes type-II hyperplasia; and H) Pulmonary pyogranuloma encased in a thick fibrous capsule, an outer mantle of lymphocytes and an inner core of macrophages, including giant cells, which have effected near complete resolution of the liquefactive center via phagocytosis of neutrophils and cellular debris. Hematoxylin and eosin staining.

bronchointerstitial pneumonia were increasingly evident with bronchiolar epithelium showing marked hyperplasia, narrowed lumina, and the replacement of peribronchiolar parenchyma by granulation tissue. Chronic active bronchointerstitial pneumonia (Figure 3.6G) described the majority of lesions in some goats as early as Day 14 and nearly all goats from Days 14 to 42. Interstitial, interlobular, perivascular, and pleural fibrosis were increasingly present, while vasculitis was less common, but was still observed in areas of active disease. Mature pyogranulomas were the most common lesion, having the typical structure (in the lung and all tissues) from inside out as follows: variably thick liquefied centers with multifocal groups of intact and degenerate neutrophils; a layer of epithelioid macrophages 5-10 cells thick with the innermost layer showing increased phagocytic activity and occasional bi- to multi-nucleate giant cells; a layer of lymphoplasmacytes arranged in concentric rows intermixed with fewer macrophages and neutrophils; and an outer encasement of granulation tissue that compresses the surrounding parenchyma. Intervening lung parenchyma showed multifocal atelectasis and perivascular lymphocytic aggregates. Resolving pyogranulomas showed increasing numbers of

macrophages and a thick mantle of lymphocytes which were in the process of eliminating the liquefied central area (Figure 3.6H). Cavitary lesions appeared to be a result of the liquefactive center retracting away from the surrounding mantel. Histologic lesions were observed in all goats except for BpG43 (Day 2 PI), which also cultured negative for *B. pseudomallei* from the lungs.

BpG42 developed fatal disease on Day 9, which was associated with an overwhelming pneumonia. Histologically disease was also notably more severe. Large abscess were surrounded by highly vascularized granulation tissue and markedly congested parenchyma with multifocal areas of hemorrhage and many vessels occluded by fibrinocellular thrombi. Abscesses were seen to multifocally erode into vessels, interlobular septa, and terminal bronchioles. Major airways and neighboring medium sized veins and arteries were largely replaced by dense aggregates of intact and degenerate neutrophils which had infiltrated the necrotic walls of affected vessels segmentally and circumferentially leading to the formation of vasocentric lesions. Walls of arteries were diffusely infiltrated by variable numbers of neutrophils which multifocally invaded necrotic outpouchings of the wall with medial hemorrhage and intimal proliferation. Many macrophages and lesser neutrophils contained large numbers of bacteria and appeared as intensely basophilic bodies.

Mediastinal and Tracheobronchial LNs: Changes at Day 2 and 7 were typically mild, with only lymphoid hyperplasia and intact neutrophils draining into the node. More advanced changes included moderate to severe lymphoid hyperplasia, capsulitis, subcapsular expansion by edema with neutrophils and macrophages, and multifocal hemorrhages in the medullary sinuses with mixed inflammation (neutrophils, macrophages, and some plasma cells). These more advanced lesions were similarly present at Day 14, along with the development of pyogranulomas. Later

time points revealed either resolution of the suppurative lymphadenitis and a return to only mild to moderate lymphoid hyperplasia, or the progressive growth and then partial resolution of pyogranulomas similar to what was observed in the prescapular LN. Rarely erythrocytes were noted entering the LN through drainage. BpG42 was observed to have bacteria laden macrophages entering the LN.

Heart: Myocarditis was observed in 94% (16/17) of goats, which ranged from minimal to moderate, focal to multifocal, random, lymphocytic to lymphoplasmacytic with rare (one goat) neutrophilic infiltrates. The interstitium was typically expanded by perivascular infiltrates of lymphoplasmacytes that followed along interstitial tissue planes (Figure 3.5C) and occasionally surrounded individual degenerate cardiomyocytes, which had fragmentation of their sarcoplasm and hyalinization with loss of cross striations. Moderate lesions also had peripheral microvascular hemorrhages and edema. Occasional Purkinje fibers were also degenerate and infiltrated by small numbers of lymphocytes. Slightly increased satellite cell activity was observed as rowing of nuclei.

Spleen: Early changes seen consisted of moderate to severe congestion and increased numbers of neutrophils in the red pulp, which rarely formed prominent perifollicular rings. This was the extent of lesions in animals that did not develop pyogranulomas. Mild extramedullary hematopoiesis was rarely noted. Progression typically involved the development of suppurative capsulitis with a variable background of edema/dilated lymphatics, vascularized granulation tissue, mesothelial hypertrophy, and leukocytoclastic phlebitis with endothelial hypertrophy. As typical pyogranulomas developed, the capsule often underwent marked thickening (5 – 20x normal) and multi focal hemorrhages were observed in the capsule. Older pyogranulomas did not have vasculitis and had the same encroachment of the outer histiocytic layer seen in the lungs

indicating early regression of lesions. The exception to this was BpG59 which had extensive pyogranulomas throughout the spleen. These pyogranulomas were noted to extend to the splenic parenchyma via necrotic trabeculae which contained similar pyogranulomas. Vascular changes were still prominent at Day 42, which included: necrotic vessels at the center of pyogranulomas and capsular vessels showing medial hypertrophy and hyperplasia. Additionally, several nerves at the periphery of resolving granulomas were surrounded by lymphoplasmacytic inflammation and occasionally infiltrated by neutrophilic exudate (abscesses) (Figure 3.5D). BpG42 did not develop pyogranulomas grossly or microscopically, but did have discrete aggregates of degenerate neutrophils (with indiscernible walls) within the parenchyma, not associated with the capsule or trabeculae.

Liver: Lesions were very common, 94% (16/17) of goats, but typically mild with no apparent association between time point and severity. Minimal to mild random hepatitis with individual hepatocyte necrosis, lymphoplasmacytic aggregates, and neutrophil infiltrates were seen at all time points except for one goat that had no hepatic changes. Moderate lesions occasionally localized around portal areas and tended to be more neutrophilic (microscopic abscesses) (Figure 3.5E). Rarely the central veins were markedly ectatic and congested with the endothelial lining disrupted as small neutrophil infiltrates extended into neighboring foci of necrotic hepatocytes. Occasional necrogranulomas were also seen scattered within the parenchyma. Centrilobular to bridging hepatic lipidosis was seen in four goats – all at Day 42. Microscopic hemorrhage was observed in two goats, associated with the capsule and minimal aggregates of neutrophils or in areas of sinusoidal edema. The old lesion noted in BpG52 was a chronic, eosinophilic granuloma not associated with *B. pseudomallei*.

Kidney: Inflammation began in glomeruli of the outer cortex (hypercellular/neutrophilic infiltrates) and then involved the tubules and intervening interstitium, expanding toward the capsule and causing suppurative capsulitis. Bordering proximal convoluted tubules were ectatic, contained luminal eosinophilic proteinaceous fluid with suspended neutrophils, and had metaplasia of the lining epithelium to low cuboidal or squamoid cells. Suppurative tubulointerstitial nephritis progressed toward the pelvis with congested capillaries, ectatic distal convoluted tubules and collecting ducts, areas of fibrin deposition, multifocal hemorrhage, leukocytoclastic vasculitis, fibrinocellular thrombi, and vascular necrosis. Suppurative inflammation eroded into the renal pelvis where the lumen was filled with large numbers of intact and degenerate neutrophils that also invaded the suburothelial stroma (Figure 3.5F). This growth created the characteristic linear structure which spanned the renal cortex, medulla, and often pelvis, replacing normal parenchyma with abundant karyorrhectic nuclear debris as an early abscess, which then matured into the typical pyogranuloma. Even as the pyogranulomas matured with increasing fibrous connective tissue, they continued to enlarge, effacing large areas of the kidney. Lesions were still very active in chronicity (Day 42), with vasculitis and suppurative pyelonephritis with visible colonies of coccobacilli. Extension into the pelvis was histologically visible in 4 of 8, likely in 1 of 8, and not observed in 3 of 8 goats with renal pyogranulomas. Varying degrees of these changes were observed in 76% (13/17) of goats, with 61% (8/13) of lesions involving culture positive pyogranulomas.

Adrenal: Minimal changes were observed in four goats independent of time point, with only three showing any suppurative component. The mildest change was a slight increase in fluid at the corticomedullary junction, which was associated with congestion and increased neutrophils

within leaky capillaries in some goats. Blood sinuses were occasionally occluded by fibrin thrombi with minimal lytic necrosis and microabscesses in the inner cortex (Figure 3.5G).

Retropharyngeal LN: Changes variably consisted of hyperplasia, edema, sinus histiocytosis, and rare hemosiderosis. A smaller pyogranuloma with a prominent macrophage capsule and lymphocyte outer jacket was present in BpG59.

Mesenteric LN: Mild to moderate suppurative capsulitis and pericapsulitis was observed in association with increased neutrophils in the medullary sinuses in BpG48. Unique lesions were also noted in BpG42, which had ectatic medullary sinuses due to edema and small hemorrhages which additionally contained many phagocytically active macrophages with intra- and extra-cytoplasmic gram negative bacteria, small numbers of neutrophils, hemosiderin laden macrophages, and plasma cells (Figure 3.5H). Changes in other goats consisted of variable hyperplasia, edema, sinus histiocytosis, and rare hemosiderosis.

Mammary Gland: Involuting or dry mammary glands without lesions were present in all female goats except BpG48, which was involuting but had a large pyogranuloma. Squamous metaplasia was present in the lining epithelium of a large duct with massive luminal aggregates of degenerate neutrophils intermixed with large colonies of coccobacilli and moderate numbers of macrophages. There was focal necrosis of the lining epithelium where neutrophils spilled into adjacent mammary tissue intermixing with large numbers of macrophages with increased phagocytic activity. The lining epithelium contained small mucosal abscesses with a cleft that separated keratin into the lumen.

Nasal Turbinates: Only the turbinates from BpG59 were examined because of its persistent shedding of *B. pseudomallei* over the latter half of the study. Neutrophils filled the lumina of several glands which were slightly hyperplastic along with goblet cells of the respiratory

epithelium. Luminal mucopurulent exudates were present with multifocal erosions and extension of inflammation into bone trabeculae and interstitial stroma. Occasional necrosis was seen in the bone trabeculae with multifocally increased osteoblastic activity.

Sublumbar LN: The sublumbar LNs were only noted to be grossly abnormal in BpG58 and were only examined in this goat. Nodes were enlarged 5x normal size with focal resolving pyogranulomas and marked expansion of subcapsular, interfollicular, and medullary sinuses with hemorrhage and erythrophagocytosis in the midst of marked follicular hyperplasia, chronic active trabeculitis, and variably severe capsulitis.

3.4i) Cytokine Response

A significant effect of day was found for TGF-β1 in both percutaneously ($p = 0.0005$) and aerosol ($p = 0.0048$) infected goats, as well as significant effects of route ($p = 0.0016$) and route x day interaction ($p < 0.0001$). There were significant decreases in TGF-β1 from Day 0 to Day 2 in both groups, but only significant elevation above Day 0 at Days 14 and 21 in the percutaneous group, with no significant difference from pre-infection levels by Day 42 (Figure 3.7A). Significant differences in the aerosol group were primarily composed of elevations at Days 7, 14, and 21 over the Day 2 trough. The concentration of TGF-β1 was significantly lower in the subcutaneous group on Days 0, 2, and 14 when compared to the corresponding days in the aerosol group. IFNγ showed a significant effect of day in percutaneously ($p = 0.0175$), but not aerosol ($p = 0.0668$) infected goats. The significance was a result of the elevation of IFNγ observed on Day 42 that was significantly higher than all other time points (Figure 3.7B).

Given the importance of CCL2 in the early immune response to *B. pseudomallei*, comparisons of Day 0 and Day 2 were made for both routes of infection, even though the overall effect of day was not significant, aerosol ($p = 0.1510$) and percutaneous ($p = 0.5582$). This

comparison showed a significant increase in CCL2 in aerosol infected ($p = 0.0273$), but not percutaneously infected goats ($p = 0.4686$) on Day 2 (Figure 3.7C). Comparisons were not made for IFNγ despite the near significance of effect of day because no similar *a priori* predictions were in place. Cytokine data are summarized in Figure 3.7, with the exception of IL-10, which did not show any significant changes in response to infection in either group.

3.5) Discussion

The route of experimental infection with *B. pseudomallei* has been repeatedly shown to be a key determinant of LD50 and disease severity. The infecting dose of *B. pseudomallei* and the duration of infection similarly affect disease presentation and outcome in experimental animals, and almost certainly play a similar role in human disease. However, the comparisons to human disease are much harder as the dose range associated with human disease is unknown and acute presentation is defined as infection ranging from one day to two months. Clinical presentation is even more variable, ranging from peracute septic shock and death to localized skin infections to chronic pneumonia and visceral abscesses, leading to the coining of melioidosis as 'the remarkable imitator' [151] as it can present as suppurative infection of virtually any organ.

3.5a) Clinical Disease

Following infection with *B. pseudomallei*, approximately 75% of goats in this study developed fever by Day 2 PI, which was followed by variable elevations in temperature for the following two weeks until temperatures generally returned to pre-infection values. This contrasts what was seen in aerosol infected goats where 100% developed fever and the mean temperature remained in the febrile range for the first nine days post infection, not returning to

pre-infection temperatures until after 14 days. This supports the findings in previous experimental models and human epidemiologic data that inhalational disease is more acute. More focused peaks of fever on Days 2 and 9 were also seen the aerosol study, while only a consistent Day 2 peak was seen in this percutaneous study [492]. This greater variability appears

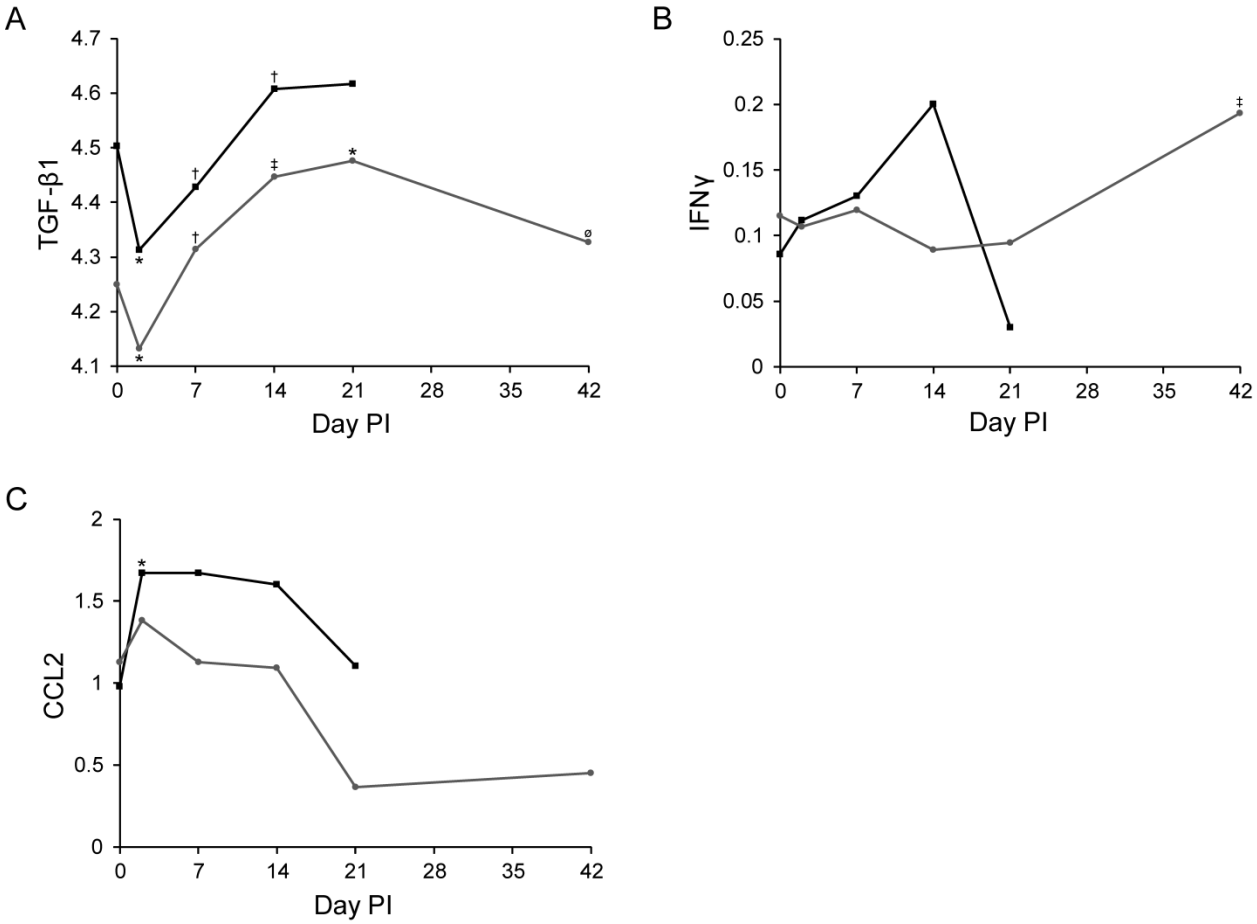

Figure 3.7: Cytokine production in goats infected with *B. pseudomallei*. Responses of A) TGF-β1 (log pg/mL), B) IFNγ (log ng/mL), and C) CCL2 (log ng/mL) in aerosol (■) and percutaneous (●) groups. Significant elevations were only present for CCL2 in aerosol infected goats and IFNγ in percutaneously infected goats. TGF-β1shows an initial decrease followed by increases through Day 21 before returning to pre-infection levels on Day 42. (‡) different than previous measure and pre-infection, (†) different than previous measure but no different than pre-infection, (*) different than pre-infection, (ø) not different than previous measure or pre-infection, significance $p < 0.05$.

route dependent, where a localized inoculum is more easily contained and may reflect individual host factors associated with the immune response compared to widespread pulmonary infection where bacterial factors dominate the presentation of initial infection. While later time points were generally not associated with fever, sporadic elevations in temperature were still observed (Figure 3.1), emphasizing the chronic active nature of the disease.

Significant correlations between granulocyte ($r = 0.26$, $p = 0.0082$) and lymphocyte ($r = -0.20$, $p = 0.0374$) counts and temperature were also seen in aerosol infected goats. Neutrophils have been shown to be critical in the host response to *B. pseudomallei* [108] such that increased granulocytes would be expected with infection, and acute elevations have been observed in both murine and NHP models [238,473]. The importance of the increasing lymphocyte count only became evident with the longer duration of the percutaneous study (42 vs. 21 day aerosol). The defervescence on Day 18 matching the onset of lymphocytosis in goats in the percutaneous study suggests the development of an adaptive immune response which was necessary to bring disease under control. Histologically, this time point was also the transition between subacute progressive disease and the development chronic disease with early signs of disease control by host immune cells.

The importance of the adaptive immune response in disease resolution (but not necessarily susceptibility) has been shown in experimental disease and melioidosis patients [325,326,330,331], though the total number of circulating lymphocytes were not investigated to compare directly to the response in goats. Acute human melioidosis has been associated with lymphopenia [496,497] and a higher total lymphocytes on Day 3 has been shown to be significantly associated with increased survival in experimental NHP melioidosis. However, evaluations at later time points have not been performed. The goat appears to be able to mount

an efficacious adaptive immune response to *B. pseudomallei*, which has been evidenced by the presence of sterile lesions in both natural and experimental disease [440,442,471]. One sterile lesion was observed in BpG58, though it did not have the typical dry, crumbly appearance noted in these earlier studies. This finding appears to be associated with lesions older than the duration of the current experiment.

The radiographic pattern and distribution of caprine pulmonary lesions in percutaneous melioidosis was typically characterized by bronchointerstitial infiltrates and pulmonary nodules, which were more prominent in the dorsal aspect of the caudal lung lobes. These findings are consistent with radiologic findings in human disease [179]. There was considerable overlap in the appearance of radiographic lesions for the two routes of infection, though aerosol infected goats typically exhibited more severe disease as judged by the size and number of pulmonary nodules. The two notable differences seen in the percutaneously infected goats – regression of lesions and the development of cavitary lesions – were likely both a function of a longer study duration, which allowed the maturation of these lesions.

Thin-walled cavitary lesions are common in human pulmonary melioidosis and similarly appear to be more associated with subacute/chronic disease [173,174,181]. Cavitary lesions have not been previously reported in goats for either naturally occurring melioidosis or previous experimental infections. The only report of cavitary lesions in animals was in sheep, where thick-walled lesions were noted, but appear to be very rare (two cases) despite the very high frequency of pulmonary lesions in sheep [105,439,441]. The lesion in BpG59 was also thick-walled (based on a thickness > 3mm [498]). It is possible that thin-walled cavitary lesions may be a unique feature of human disease, but studies of longer duration may yield thinner-walled

lesions in goats as there will be more retraction of the central core and thinning of the surrounding capsule with maturation.

Human patients with radiographic evidence of acute pulmonary melioidosis often show patchy alveolar infiltrates and nodular lesions with rapid radiographic and clinical progression, which is frequently fatal [183,421]. This was similar to the disease in BpG42, which had moderate patchy bronchointerstitial and alveolar infiltrates and nodules on Day 7, which rapidly progressed to diffuse disease and death on Day 9.

3.5b) Pathogenesis

Marked suppurative inflammation developed subsequent to percutaneous inoculation. Rapid and distant dissemination was evident by Day 2 with positive cultures in the prescapular LN, lungs, and spleen. The most likely path of spread was through draining ectatic lymphatics to the prescapular LN and then through efferent lymphatics to the blood stream and distant organs. Vasculitis at the site of infection also could also allow direct hematogenous invasion and dissemination of *B. pseudomallei* in addition to lymphatic-hematogenous dissemination.

The association of vasculitis with lesion development in the lungs, spleen, kidneys, and lymph nodes and frequent observation of vasocentric abscesses within the lungs strongly supports hematogenous dissemination. Uniformly negative blood cultures, despite the collection of larger blood volumes, highlights the low levels and intermittent bacteremia responsible for dissemination in goats. Dissemination to the kidney with the first days of infection was also indicated by the presence of a grossly visible abscess on Day 7. Previous experience has shown that abscesses require about seven days from the time of dissemination to enlarge enough to become visible grossly.

The dissemination was more rapid than in aerosol infected goats, which typically did not show extrapulmonary spread before Day 14. Additionally, the histologic appearance of lesions was often more severe than the lesions observed following aerosol infection [492]. Hemorrhage was more frequently observed both directly in tissues as well as in lymph nodes that were draining areas of hemorrhage following percutaneous infection, whereas hemorrhage was primarily associated with severe, fatal disease after aerosol infection. Despite the more aggressive nature of lesions, there were not dramatic increases in morbidity and mortality associated with higher doses (up to 8.2×10^6 CFU) in pilot percutaneous infection studies (data not shown), which were seen in aerosol studies of comparable doses.

Even though bacteria were inoculated percutaneously, there was a very strong predilection for the respiratory tract, with very high rates of pulmonary and tracheal involvement. Other than a slightly higher frequency of vasocentric abscesses and pyogranulomas, respiratory lesions were virtually identical to lesions observed previously in aerosol-infected goats. This included lymphoplasmacytic to erosive suppurative tracheitis with mucosal abscess formation and necrosuppurative bronchiolitis with necrotic epithelium and neutrophilic aggregates. Disease localizing to the airway was also evident in the radiographic findings, which were predominated by bronchointerstitial infiltrates.

Early lesions in all organs were dominated by neutrophils, but rapidly progressed to mixed infiltrates mostly composed of macrophages, and lymphoplasmacytes and then pyogranulomas with varying degrees of granulation tissue formation and fibrosis. This progression of cell type is similarly observed in murine models [285,462]. These early changes are also associated with changes in the inflammatory cytokine CCL2, which is typically associated with macrophage infiltration and significant increases have been observed in acute

melioidosis models (*in vitro* and *in vivo*). However, there may be an effect of route, with increases seen with aerosol infection [486], no significant changes with intranasal infection [288], and a decrease in transcription following intravenous infection [238]. The effect could be related to bacterial delivery to the pulmonary epithelium, which produces CCL2 in response to *B. pseudomallei* [499]. This may explain why a significant increase in CCL2 was seen in aerosol infected goats on Day 2, but no such changes were found in percutaneously infected goats.

Increases in serum IFNγ are also seen in murine models of acute infection – typically associated with higher organ burdens [288,481], as well as in NHP models [473]. A significant increase in chronic disease (22 – 45d PI) has also been shown in a murine model, which was associated with chronic active disease without bacteremia [483]. The increase in IFNγ observed in percutaneously infected goats mirrored this finding in chronic disease, though no significant increase in serum IFNγ was observed acutely. This was likely a result of the less acute disease/lower dose at Day 2. The elevated serum IFNγ may have also related to the extensive splenic and renal abscessation present in several goats on Day 42 and/or the release from inhibition by TGF-β1, which had decreased to pre-infection levels by Day 42.

The significance of the observed changes in TGF-β1 are less clear as few studies have examined its role in natural or experimental disease. Additionally, TGF-β1 can function as an anti-inflammatory or pro-inflammatory molecule such that it is still unclear if its elevation during disease is acting to limit immune pathology or promote inflammatory immune responses to facilitate bacterial clearance. TGF-β1 has been shown to be elevated in active cases of human melioidosis and return to normal levels after recovery [289]. It is also elevated in acute disease in mice, but may not play a major role in disease as its neutralization decreases the bacterial load in the lungs and blood, but did not change the time to death [289]. The changes observed in

goats could be associated with an initial consumption of latent (measured) TGF-β1 and a lag in production, which would explain the Day 2 trough (Figure 3.7A). The subsequent increase corresponded with the period of most active disease followed by a decline at Day 42 when disease is more chronic and less active suggesting an immunosuppressive/regulatory role for TGF-β1 in caprine melioidosis. The significant effect of inoculation route appears to most likely relate to a difference in the goat groups, rather than a true route effect. Similarly, the day x route interaction, while statistically significant, does not appear practically important as the changes in TGF-β1 appear essentially parallel.

Elevated IL-10 has been found to be significantly associated with mortality in melioidosis patients, with a 10-fold elevation in non-survivors [290]. Several murine models (acute and chronic) have not been able to reproduce this finding [483,500,501], though one study has demonstrated a significant change in IL-10 over the course of infection, with the largest elevation appearing at Day 2 [486]. Significant changes in IL-10 were not observed in the caprine models likely because of the target of subacute to chronic, non-fatal disease.

3.5c) Comparative Disease

Pneumonia is the most common presentation of melioidosis, with just over 50% of patients presenting with primary pneumonia and another 8% having secondary pneumonia [8]. The 100% occurrence of pulmonary lesions in experimental aerosol and inhalational disease is expected given the route of infection and typically high doses. A lower percentage of percutaneously infected goats were expected to have pulmonary lesions given the route and dose. Even though *B. pseudomallei* is known to have a predilection for the lungs, regardless of the route of infection, less than 50% of goats could have been expected to have pulmonary disease since at least a portion of human disease is likely from inhalational infection which was not a

factor in this study. The pulmonary lesions were quite small in several goats at necropsy, but were all clinically detectable with some degree of radiographic evidence of disease. It is possible that the rapid development of pulmonary lesions seen following percutaneous infection may lead to the diagnosis of a primary pneumonia in human melioidosis patients that is actually secondary to inoculation and hematogenous spread. The occurrence of a cavitary pulmonary lesion in one goat is notable, but it is difficult to make many parallels to human disease beyond the radiographic finding, which was not visible by *in vivo* radiography (but would have been visible by computerized tomography, which is frequently used in human patients). It is not possible to compare the histologic description of cavitary lesions because no description of the histology of human cavitary melioidosis lesions exists. The pathologic description of these lesions is limited to Whitmore's original case series in 1913 [9].

Throat swabs in human melioidosis patients (the closest approximation for nasal swabs in goats) have a sensitivity of 36% [338], which is very similar to the 35% of goats that were positive by nasal swab in this study. This suggests possible spread of infection between animals, but horizontal transmission of disease is exceedingly rare in human disease [14]. The frequency of positive urine culture in percutaneously infected goats (29%) was also similar to the incidence in human melioidosis (21-23%) [144,185]. The number of bacteria isolated from the urine was quite variable (Table 3.2) but comparable to quantitative cultures from human patients [185]. Positive urine culture was associated with advanced disease in goats, with four of five positive cultures found on Day 42 and the fifth positive from the goat with terminal disease. This association relates well to the finding that positive urine culture is a negative prognostic indicator in human disease [144], which could similarly relate to advanced disease.

The involvement of other organs was generally similar to that seen in human and other experimental disease models with the exception of gross liver lesions, which was previously noted in our aerosol infection model [492]. Splenic capsulitis was common as in the aerosol infection model, with the additional finding of splenic neuritis, which could be a source of the abdominal pain observed in human patients [58,188]. A novel finding in the caprine percutaneous infection model was the presence of microscopic cardiac lesions of interstitial myocarditis. The lesions were highly consistent, but never severe enough to cause clinical manifestations. Macroscopic cardiac lesions have been reported as a rare finding in one study of naturally occurring disease in a group of debilitated goats [443]. Gross [502,503] and microscopic [420,422] myocardial lesions have been reported in fatal human cases of melioidosis, but cardiac lesions are very rare (<1%) and typically involve pericardial disease [8].

The other highly consistent finding was abscessation of the draining lymph node. This has been found in previous caprine models [470,471], but is only reported in 2% human melioidosis patients [8]. Of the patients with suppurative lymphadenitis, the majority of lymph nodes involved were in the head and neck, with only one report documenting inguinal lymph node involvement [504]. A higher incidence of inguinal lymph node involvement would be expected since many rice farmers are believed to be infected percutaneously through cuts on their feet. Natural disease in goats has also been shown to involve lymph nodes, but the most commonly affected are the mediastinal lymph nodes draining the lungs [105,439,442]. Prescapular lymph nodes (draining the forelimb) have been found to be affected in naturally infected goats as well, but much less frequently [440,441,445]. This suggests either that percutaneous inoculation is actually an uncommon cause of natural infection or much smaller inocula are involved with natural disease such that significant enlargement and abscessation of

the lymph node does not occur. In pilot studies of percutaneously infected goats, the infecting dose seemed to have the greatest effect on abscessation at the site on infection and the draining lymph node, but relatively little effect on the development of lesions at more distant sites such as the lung, spleen, and kidney.

The factor that seemed to have the greatest impact on disease outcome was the health status of the individual goat. Most goats were able localize infection to abscesses and isolate it further with the maturation of pyogranulomas. The establishment of these types of lesions is in and of itself viewed as an indication of a more successful immune response [58]. In this study, only BpG42 progressed to fatal disease, unable to localize the infection as seen in numerous culture positive organs and severe miliary pneumonia. Similar fatal disease was seen in one pilot study goat which had a thin body condition (similar to BpG42) and anemia. While BpG42 was not anemic, it did have a mild elevation in granulocytes prior to infection. These findings suggest some underlying disease, which, while mild in clinical appearance, appeared to significantly affect the outcome of disease. Initial disease progression in these animals appeared similar to the rest of the group, but instead of a transition to chronic disease, rapidly progressed to fatal disease. The gross, histopathologic, and radiographic changes associated with these lesions were similar to acute fatal cases of human melioidosis [421,423]. The importance and interplay of individual host factors are well-established in human disease [8], and may even supersede the importance of dose or the route of infection in human disease.

The strength of a caprine model is the ability to compare experimental and natural disease in a species that lives within the endemic range of melioidosis, and is epidemiologically similar to humans. The findings presented here provide a detailed clinical, radiographic, and pathologic description of the pathogenesis of percutaneous caprine melioidosis, which compares favorably

with human disease. The goat appears well-suited for the study of subacute to chronic disease as well as the incorporation of risk factors to model acute human melioidosis.

CONCLUDING REMARKS

The basic thrust of this dissertation research was model development and study of pathogenesis, which is a holistic process requiring a thorough understanding of the disease as it occurs in the human population, the pathogen, the host species, and the manipulatable factors of their interaction that will produce a desirable model. Melioidosis is a highly variable disease with diverse presentations such that it is very difficult to model, since the goal of a model is to have consistent, reproducible disease. It is impossible to have an 'ideal' model of melioidosis since an extremely consistent model will be unrealistic and a highly variable model will not be useful. The focus must therefore be on understanding the strengths and weaknesses of different model systems and what can or cannot be usefully determined from them. The end result is hopefully the use of multiple animal models, which will create a more complete and robust understanding of melioidosis through comparative study.

We strived to develop the caprine models to balance the competing needs of repeatable and relevant disease. However, the limitations of the models must be acknowledged alongside their strengths. The fact that the goat is a naturally affected species with similar epizootiology/ epidemiology and clinical disease compared to humans is a particular strength of the model. A notable exception that remains to be resolved is the central nature of vasculitis to the pathogenesis of caprine melioidosis, which is unreported in human cases. The immunologic and genetic tools available to study the molecular pathogenesis in goats is currently very limited such that a detailed examination of some of the processes is simply impossible in goats, though the increasing accessibility of next generation sequencing technology may eliminate some of these barriers. The large body size of goats does present a unique opportunity for serial diagnostics, but there are few facilities than can conduct research with *B. pseudomallei* in large animals.

However, relative to NHPs, it is comparatively easy and certainly less expensive to work with goats. The outbred nature of goats ensures a heterogeneous population for infection studies, which is similar to people, but also requires larger sample sizes.

Evaluating the models in hindsight, the one change to most improve their utility would be using a natural (nasal) inhalational method of exposure for aerosol infection rather than intratracheal delivery. The latter allows for more controlled delivery, but bypasses the entire upper respiratory tract which is central to pathogen entry and the mucosal immune response. It also likely explains why no animals developed neurologic signs, which has been repeatedly observed in goats from both Australia [105,439] and Malaysia [443]. Direct invasion of the central nervous system through the olfactory epithelium and nerve was not possible with this route of infection, and later colonization of the nasal cavity appeared insufficient to cause neurologic lesions.

The apparent ability of goats to survive acute infection and possibly cure chronic infection provides an opportunity to examine what epitopes are involved in a successful immune response to melioidosis such that they can be targeted by vaccination or therapeutic measures. The adaptations of *B. pseudomallei* that occur as a result of this immune response – latent infection or the development of a viable but non-culturable state – could also be studied using caprine models and provide insights into the difficulties of treating and curing melioidosis. The last advantage in regards to disease modeling is the fact that goats appear to have 'risk factors' that predispose them to severe disease. While this was noted in very few animals, it is a very important observation since healthy individuals are generally resistant to natural melioidosis, certainly in regards to acute severe disease, while individuals with predisposing factors are at much greater risk.

The final consideration that should be made of the goat model is its broader application to human health. Goats are an important domestic species in much of the developing world where melioidosis is endemic. Enzootic and epizootic disease not only poses a health risk from saprozoonotic infection, but also can cause economic impacts as well. Any knowledge gained from the use of these models in regards to improved diagnosis, preventive measures, and vaccination would be directly applicable to the management of these domestic goat populations, which can provide a direct benefit to people beyond just a greater understanding of disease.

In addition to a detailed clinical, radiographic, and pathologic description of the pathogenesis of subacute to chronic aerosol and percutaneous caprine melioidosis, several important novel aspects of disease have been revealed. Percutaneous infection can produce disease that disseminates as rapidly, if not more so, than aerosol infection, with both similarly affecting the lungs in the temporospatial development of lesions. The histologic appearance of lesions as well as the rapid dissemination supports percutaneous exposure as an important route of infection. However, the role of oral infection is in need of further investigation. This is clearly complicated in a ruminant model, but the dissemination to the mesenteric lymph node seen in fatal disease in the absence of gross gastrointestinal lesions is intriguing. The severity of disease produced in healthy animals along with its clinical features suggests that infecting doses in people may be quite low.

The findings presented provide clear support for the use of caprine models in the study of melioidosis as well as a strong foundation of knowledge of disease pathogenesis for further model refinement and application as we move forward with comparative investigations of this serious emerging infectious disease.

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LIST OF ABBREVIATIONS

ABSL-3	Animal biosafety level-3
AHL	N-acyl-homoserine lactones
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CBC	Complete blood count
CCL2/MCP-1	Chemokine (C-C motif) ligand 2 /monocyte chemotactic protein-1
CCL3/MIP-1α	Chemokine (C-C motif) ligand 3/Macrophage inflammatory protein-1α
CCL4/MIP-1β	Chemokine (C-C motif) ligand 4/ Macrophage inflammatory protein-1β
CCL5/RANTES	Chemokine (C-C motif) ligand 5/regulated and normal T cell expressed and secreted
CD	Cluster of differentiation
CFU	Colony forming unit
CIS	Cytokine-inducible Src homology 2-containing protein
CL	Caseous lymphadenitis
CNS	Central nervous system
CR	Computed radiography
CXCL1/KC	Chemokine (C-X-C motif) ligand 1/Keratinocyte-derived Cytokine
CXCL2/MIP-2α	Chemokine (C-X-C motif) ligand 2/Macrophage inflammatory protein-2α
CXCL3/MIP-2β	Chemokine (C-X-C motif) ligand 3/Macrophage inflammatory protein-2β
CXCL9/MIG	Chemokine (C-X-C motif) ligand 9/Monokine induced by gamma interferon
CXCL10/IP-10	Chemokine (C-X-C motif) ligand 10/Interferon gamma-induced protein 10
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
ICT	Immunochromatographic test
IFNγ	Interferon gamma
IHA	Indirect hemagglutination assay
IL	Interleukin
IN	Intranasal
IP	Intraperitoneal
IT	Intratracheal
IV	Intravenous
LAP	LC3-associated phagocytosis
LN	Lymph node
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MedLN	Mediastinal lymph node
MesLN	Mesenteric lymph node

MH	Muller-Hinton
MNGC	Multinucleate giant cell
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NLR	Nucleotide oligomerization domain (NOD)-like receptors
NOS2	Nitric oxide synthase 2
ODN	Oligonucleotides
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Post infection
PO	*Per os*
PSLN	Prescapular lymph node
qPCR	Quantitative real-time polymerase chain reaction
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
RPLN	Retropharyngeal lymph node
RPM	Revolutions per minute
SARM	Sterile-α and armadillo motif-containing protein
SC	Subcutaneous
SOCS	Suppressor of cytokine signaling
SPS	Sodium polyanethanol sulfonate
T3SS	Type three secretion systems
T6SS	Type six secretion systems
TBLN	Tracheobronchial lymph node
TGF	Transforming growth factor
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha