Differences in Inflammation Patterns Induced by African and Asian *Burkholderia pseudomallei* Isolates in Mice

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Abstract. *Burkholderia pseudomallei* is the causative agent of melioidosis, an emerging tropical disease of high mortality. Sub-Saharan Africa represents potential melioidosis “hotspots”; however, to date, only a few cases have been reported. Here in, we compared the inflammatory patterns induced by a *B. pseudomallei* strain recently isolated from a fatal Gabonese case with the Thai reference strain *B. pseudomallei*-1026b and *Burkholderia thailandensis*-E264. Ex vivo, no differences were observed in terms of cellular responsiveness between strains. However, when compared with the *B. pseudomallei*-1026b strain, the Gabonese isolate was significantly less virulent in terms of bacterial dissemination, inflammatory response, and organ damage in mice. Genomic comparison between strains showed differences in regions containing a fimbriae/adhesion virulence protein. In addition to a lack of microbiology facilities, differences in virulence of *Burkholderia* strains might contribute to the diverse global clinical occurrence of melioidosis.

The Tier 1 biothreat agent *Burkholderia pseudomallei* is an environmental Gram-negative bacillus and the causative agent of melioidosis, a tropical infectious disease classically characterized by pneumonia and abscess formation throughout the body.^{1–3} The *Burkholderia* genus contains over 40 species, of which *B. pseudomallei* and *Burkholderia mallei* are considered the most pathogenic.^{1,4} *Burkholderia thailandensis* is closely related to *B. pseudomallei* but rarely causes disease.⁵ *Burkholderia pseudomallei* governs an impressive arsenal of virulence factors, including the capsular polysaccharide, lipopolysaccharide (LPS), type-III secretion systems, flagella, fimbriae, adhesion virulence proteins, and the toxin named BPSL1549 *Burkholderia* lethal factor-1.^{6,7} However, the relative impact of each on clinical outcome in human infection remains largely unknown. One could hypothesize that potential differences in the virulence of *B. pseudomallei* strains explain in part differences in clinical occurrence of melioidosis around the globe.

Melioidosis is endemic in northern Australia and south-east Asia with annual incidence rates of up to 50 patients per 100,000 people.^{1,8} There are about 165,000 human melioidosis cases per year worldwide, of which 89,000 people succumb to their disease.⁹ It is estimated that melioidosis is widespread in sub-Saharan Africa with 24,000 cases of melioidosis occurring each year associated with a predicted mortality rate of 62.5%.⁹ However, less than 20 cases of melioidosis from Africa have been reported. In addition to severe underreporting as well as a lack of diagnostic facilities,^{10,11} differences in the virulence of *Burkholderia* strains could explain this disparity.

Therefore, in the present study, we determined the differences in patterns of virulence of a recently isolated *B. pseudo-*

mallei strain from a fatal Gabonese case by comparing it to the virulence of the well-typed virulent Thai isolate-1026b and the nonpathogenic *B. thailandensis*-E264 by investigating the host response to these *Burkholderia* strains in mice ex vivo and in vivo.^{4,12,13} In addition, we compared whole genome sequences on the presence of potential virulence factors of both strains.

Male wild-type C57Bl/6 mice (Charles River, Leiden, the Netherlands) 8–10 weeks of age were used. *Burkholderia pseudomallei*-1026b strain has been isolated from a blood culture of a septic 29-year-old Thai female rice farmer presenting with bacteremia with soft tissue, skin, joint, and splenic involvement.¹³ The Gabonese *B. pseudomallei* isolate was isolated from a blood culture of a 68-year-old diabetic septic female with soft tissue and joint involvement.¹² *Burkholderia thailandensis*-E264 is an environmental isolate from northeast Thailand.⁵

Murine whole blood, peritoneal macrophages, and alveolar macrophages were isolated as described^{4,14,15} and stimulated overnight with medium, *Escherichia coli* LPS, or *Burkholderia* isolates. Pneumonia was induced by intranasal inoculation with 7.5×10^2 colony-forming units (CFU) of *B. pseudomallei*-1026b, the Gabonese *B. pseudomallei* isolate, or *B. thailandensis*-E264. Sample harvesting, processing, and determination of bacterial growth, assays, and pathology were done as described.^{4,14,15} Whole-genome sequencing was performed using the MiSeq platform (Illumina, San Diego, CA) as described.^{11,12} Genome alignments and annotation of *B. pseudomallei*-1026b strain (NC_017831.1 and NC_017832.1) were obtained from GenBank.¹⁶ For full methods see Supplemental Information.

To investigate the differences in inflammation induced by infection with the Gabonese *B. pseudomallei* isolate and *B. pseudomallei*-1026b, we first stimulated different murine cell types considered important for the regulation of inflammation in melioidosis. In terms of pro-inflammatory cytokine release, we observed no differences between the Gabonese isolate when compared with *B. pseudomallei*-1026b in whole

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blood (Figure 1A), peritoneal macrophages (Figure 1B), or alveolar macrophages (Figure 1C). Interleukin (IL)-6 levels were comparable to tumor necrosis factor (TNF)- α (data not shown).

In vivo, however, mice infected with 7.5×10^2 CFU of the Gabonese *B. pseudomallei* isolate showed markedly decreased bacterial loads in lung, liver, and blood 72 hours after intranasal inoculation when compared with the groups infected with 1026b strain (Figure 1D–F). Interestingly, bacterial loads of mice infected with the Gabonese *B. pseudomallei* isolate were similar to those in mice infected with the aviru-

lent *B. thailandensis*. At this dose, none of the eight blood cultures of mice infected with the Gabonese *B. pseudomallei* isolate became positive. Only intranasal inoculation of the Gabonese isolate with a dose that was seven times higher than the *B. pseudomallei*-1026 dose resulted in equal bacterial loads in lung, liver, and blood between both strains 72 hours after infection (Figure 1D–F).

Local and systemic inflammation, as assessed by determination of chemokine (monocyte chemotactic protein-1) and cytokine (IL-6, IL-10, interferon- γ , and TNF- α) levels, was

FIGURE 1. Comparative virulence of the different *Burkholderia* strains ex vivo and in vivo. (A) Whole blood, (B) peritoneal macrophages (PM), (C) and alveolar macrophages (AM) were isolated from naïve wild-type C57BL/6 mice and were stimulated overnight with medium, lipopolysaccharide of *Escherichia coli* (100 ng/mL), heat-killed *Burkholderia pseudomallei*-1026b (B.ps), or the Gabonese *B. pseudomallei* isolate (B.gab). After 20 hours of stimulation, supernatants were harvested and assayed for membrane-associated tumor necrosis factor- α (mTNF α); ($N = 4$ per group). Additionally, mice were infected with 7.5×10^2 colony-forming units (CFU) of *B. pseudomallei*-1026b (B.ps), *Burkholderia thailandensis* (B.th), or the Gabonese *B. pseudomallei* isolate (B.gab) and were killed at 72 hours postinfection and bacterial loads were determined in (D) lung, (E) whole blood, and (F) liver. The dashed line marks the level of CFU detection. None of the eight blood cultures were positive for mice infected with the Gabonese *B. pseudomallei* isolate (B.gab). For the dose-finding experiment, mice were infected with 10^2 , 10^3 , or 5×10^3 CFU of the Gabonese *B. pseudomallei* isolate (B.gab) and killed 72 hours postinfection. $N = 8$ per group. Kruskal–Wallis test was performed, followed by post hoc Dunn's test, * $P < 0.05$, *** $P < 0.001$.

significantly reduced in mice infected with the Gabonese *B. pseudomallei* isolate when compared with the 1026b strain (Supplemental Table 1). Next, we determined the severity of organ inflammation in all groups. Mice infected with the Gabonese *B. pseudomallei* isolate showed markedly attenuated pulmonary and liver injury when compared with the strain 1026b (Figure 2). Evidence of profound organ damage was further reflected in increased alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) levels 72 hours after infection in all mice (Supplemental Table 1). However, in line with the pathology data, mice infected with the Gabonese *B. pseudomallei* showed strongly reduced ALT, AST, and LDH levels (Supplemental Table 1). In addition, we observed lower creatinine and blood urea nitrogen levels in Gabonese *B. pseudomallei*-infected mice, possibly due to better renal perfusion, since these mice appeared less septic (Supplemental Table 1). The inflammatory response elicited by the Gabonese *B. pseudomallei*, as determined by local and systemic cytokine production as well as pathology and markers of organ damage was comparable to that of mice infected with *B. thailandensis* (Supplemental Table 1).

To find the possible underlying cause of the observed decreased virulence of the Gabonese *B. pseudomallei* isolate when compared with *B. pseudomallei*-1026b, we used the Basic Local Alignment Search Tool algorithm to find regions unique to *B. pseudomallei*-1026b. The Gabonese *B. pseudomallei* isolate showed 13 chromosomal putative protein regions (A–M) that corresponded poorly to those of strain 1026b (Figure 3, Supplemental Table 2). Within these regions, most annotations classify the putative proteins into phage, integrase, transposase, and hypothetical proteins.

Genomic inspection of the found regions, on potential causal proteins, showed one region, namely E (Figure 3), containing a fimbrial and adhesion virulence protein (Type IV fimbrial biogenesis protein PilY1).¹⁷

Our experiments demonstrated that the Gabonese *B. pseudomallei* strain is just as virulent as the reference strain *B. pseudomallei*-1026b ex vivo but, more importantly, less virulent in vivo. This might in part be explained by the more complex interaction between host and bacterium in an in vivo model. In addition, the in vitro inflammatory response demonstrates only a portion of the complete immune response.

The majority of previously published studies about murine melioidosis are conducted with the “prototypical” strains 1026b or K96243 (isolated from 34-year-old Thai diabetic female).¹³ We conducted in vivo survival studies, which demonstrated equal lethality of both strains, when the same inoculation dose was used (data not shown). Following these experiments, we chose to compare the well-typed 1026b strain with the recently discovered *B. pseudomallei* isolate of a Gabonese patient. However, it is important to keep in mind that the pathogenicity of *Burkholderia* strains varies in animal models¹⁸ and does not always correlate with their virulence in humans, depending on host factors.¹⁹

It is known that “serial passaging,” a transfer of a bacterium through a series of cultures or via experimental animals, may increase virulence of bacterial strains by selecting the most virulent bacteria²⁰. In addition, it is known that *B. pseudomallei* can rapidly genetically modify itself within the host.²¹ Nonetheless, the virulence of the Gabonese *B. pseudomallei* isolate did not differ before and after passaging, thereby

FIGURE 2. Diminished pulmonary and liver inflammation in mice infected with the Gabonese *Burkholderia pseudomallei* isolate. Mice were infected with 7.5×10^2 colony-forming units of the Gabonese *B. pseudomallei* isolate (*B.gab*), *B. pseudomallei*-1026b (*B.ps*), or *Burkholderia thailandensis* (*B.th*). (A, E) Seventy-two hours postinfection, pulmonary and hepatic injury and inflammation were assessed by calculating pathology scores. Representative slides of hematoxylin and eosin–stained lungs and livers of mice infected with (B, F) *B. pseudomallei* (*B.ps*), (C, G) *B. thailandensis* (*B.th*), or (D, H) *Burkholderia gabonensis* (*B.gab*) are shown (original magnification 10 $\times$). $N = 8$ per group. Kruskal–Wallis test was performed, followed by post hoc Dunn’s test, $^{**}P < 0.01$, $^{***}P < 0.001$. The arrow indicates manifest inflammation.

FIGURE 3. Genome comparison of the Gabonese *Burkholderia pseudomallei* isolate with the reference strain *B. pseudomallei*-1026b: depiction of unique regions. Thirteen chromosomal putative protein regions (A–M) of *B. pseudomallei*-1026b strain that have low correspondence are compared with the Gabonese *B. pseudomallei* genome. Within these regions, most annotations classify the putative proteins into phage, integrase, transposase, and hypothetical proteins. Genomic inspection of the found regions, on potential causal proteins, showed one region, namely E, containing a fimbrial and adhesion virulence protein (Type IV fimbrial biogenesis protein PilY1). Unique *B. pseudomallei* regions are shown with the location, length, and number of proteins.

remaining significantly less virulent than the 1026b strain. See Supplemental Information and Supplemental Figure 1 for more details.

Our dose-finding experiments demonstrate that a 7-fold higher dose of the Gabonese isolate is required to be equally virulent to the 1026b strain. It should be noted that the supposedly “avirulent” *B. thailandensis* can become as lethal as the 1026b strain when a 1000-fold higher dose is used in mice, demonstrating the potential harmfulness of even this “nonvirulent” bacterium.⁴ We would like to stress that, in this study, we only compared a single African isolate with a single Asian isolate. In theory, one could hypothesize that, to cause melioidosis in humans, Gabonese isolates have to infect their host at a higher concentration when compared with their Asian counterparts. This could, in addition to severe under-reporting, explain in some part the low number of melioidosis cases reported from Gabon and Africa.

Clinical *B. pseudomallei* isolates may have a broad genomic diversity.^{13,21} In a previous analysis, a large number of *B. pseudomallei* isolates from Thailand and Australia showed a strong genetic differentiation based on geographical location and significant differentiation based on virulence potential.²² Recently, it has been shown that African strains have substantial genetic diversity, suggesting long-term *B. pseudomallei* endemicity in this region.²³ We compared the genomes of the Gabonese and the *B. pseudomallei*-1026b strain and identified a region that contains a fimbrial and adhesion virulence protein (Type IV fimbrial biogenesis protein PilY1), which was absent in the Gabonese strain.¹⁷ Variation in this region might be responsible for observed differences in virulence between the African and Asian strain. However, a larger analysis on the in vivo virulence and genetic expression between African and Asian strains is required to further address this hypothesis.²⁴

In conclusion, we found that the Gabonese *B. pseudomallei* isolate was significantly less virulent compared with a well-defined Thai isolate 1026b. This study is the first to compare the virulence of an African and Asian *B. pseudomallei* strain both ex vivo and in vivo, thereby including genomic

comparison. Recently, it has been predicted that the global burden of melioidosis is much higher than previously thought, with a worldwide mortality comparable with measles, dengue, and leptospirosis.⁹ This study highlights the need for increased insight into the virulence and expression patterns influencing pathogenicity of *B. pseudomallei* isolates across the world.

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