

Burkholderia pseudomallei Lipopolysaccharide Genotype Does Not Correlate With Severity or Outcome in Melioidosis: Host Risk Factors Remain the Critical Determinant

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Background. The causative agent of melioidosis is the Gram-negative bacterium *Burkholderia pseudomallei*. Clinical presentations of melioidosis are notably diverse, with host risk factors considered central to progression from infection to disease and clinical outcome. Ubiquitous and variably present virulence determinants have been described for *B pseudomallei*, with several variably present minority genotypes associated with specific disease presentations. The lipopolysaccharide (LPS) O-antigen of *B pseudomallei* is highly diverse with 3 types described. In vitro data suggest differential virulence between LPS types, but it remains unclear whether this LPS O-antigen diversity influences clinical presentation, severity, and outcomes in patients with melioidosis.

Methods. Whole-genome sequencing was performed to assign an LPS type to 1005 consecutive *B pseudomallei* strains, each corresponding to a melioidosis patient enrolled in the 28-year Darwin Prospective Melioidosis study. Correlations of LPS genotype with clinical parameters was then undertaken.

Results. Bivariate analysis demonstrated that mortality and the rates of bacteremia and septic shock were the same for patients with the 2 predominant *B pseudomallei* LPS genotypes A (87% of cases) and B (12% of all cases). Mortality was 12% and 12%, bacteremia was 57% and 53%, and septic shock was 22% and 18% for LPS A and LPS B, respectively.

Conclusions. Lipopolysaccharide genotype was not associated with melioidosis severity or outcome. These findings suggest that in vitro differential virulence between *B pseudomallei* LPS genotypes does not translate to clinical significance, and this supports the primary role of host risk factors in determining disease severity and outcomes in melioidosis.

Keywords. LPS O-antigen diversity; melioidosis; virulence.

The formidable pathogen *Burkholderia pseudomallei* is a Gram-negative bacillus present in soil and water in endemic regions [1]. Regions of endemicity continue to be unveiled, with *B pseudomallei* now considered endemic in Southeast and South Asia, tropical northern Australia, locations in the Americas and Africa, and numerous islands in the Indian and Pacific Oceans [2–6]. *Burkholderia pseudomallei* possesses an impressive ability to infect humans and a wide range of animals, resulting in either asymptomatic infection or the clinical disease melioidosis, which is a major cause of community-acquired pneumonia

and sepsis in northern Australia and Northeast Thailand [7, 8]. *Burkholderia pseudomallei* is a Tier 1 select agent in the United States (<http://www.selectagents.gov/>), with consequent extensive studies resulting in a greatly expanded understanding of the pathogenesis of melioidosis, including, host responses and specific *B pseudomallei* virulence mechanisms [9–14].

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria. The *B pseudomallei* LPS has been classified as a type II O-polysaccharide (O-PS; *wbi* gene cluster) and is one of a number of important surface polysaccharides in this pathogen [15]. Structurally, LPS is composed of an outer unique O-antigen and an inner core oligosaccharide that is linked to a lipid A. The LPS O-antigen moiety is structurally diverse, and for *B pseudomallei* it is grouped by serotype into types A, B, and B2 [16]. LPS type A is the most abundant O-antigen in Thai (~97%) and Australian (~85%) *B pseudomallei* [16]. A small number of isolates from Australia and Asia have LPS type B, whereas LPS type B2 is the rarest LPS type in Australia and has yet to be identified in Asian strains [16]. It is presently unknown what LPS types predominate in other endemic regions globally.

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LPS plays a critical role in stimulating the host innate immune response during melioidosis infection, with *B pseudomallei* LPS required for serum complement resistance and virulence [15, 17–19]. Previous studies have demonstrated that LPS O-antigen mutants are markedly attenuated in animal models of disease [18, 20] and are more susceptible to macrophage killing during early stages of infection [17]. The objective of this study was to assess whether a given LPS type (LPS A, B, or B2) is correlated with melioidosis disease severity (bacteremia, septic shock, or death) or clinical manifestations. To examine whether LPS diversity is associated with melioidosis severity or clinical manifestations, we determined the LPS O-antigen type from the initial *B pseudomallei* isolate from 1005 consecutive patients with a bacterial isolate in the Darwin Prospective Melioidosis study [7] and then correlated these with melioidosis clinical manifestations, severity, and outcome.

METHODS

Ethics Statement

This study was approved by the Human Research Ethics Committee of the Northern Territory Department of Health and the Menzies School of Health Research (HREC 02/38).

Clinical and Epidemiological Features of 1005 Confirmed Melioidosis Cases

The Darwin Prospective Melioidosis study [7] from the tropical north of the Northern Territory of Australia commenced in October 1989, and primary *B pseudomallei* isolates were available for 1005 patients. Each of the patients was categorized into 1 of 9 primary diagnoses, reflecting the predominant clinical features on presentation: pneumonia, genitourinary involvement, blood culture positivity but no identifiable focus, localized skin infection without sepsis, neurological melioidosis, internal soft tissue abscess, osteomyelitis, septic arthritis, and other. Bivariate disease severity metrics were also included: blood culture positivity, septic shock, and mortality. Gender, ethnicity (Indigenous Australian or not), and age (as a continuous variable) were recorded. In addition, each patient was scored as absent or present for history of an inoculation event and for 8 melioidosis risk factors: hazardous alcohol use, diabetes, chronic renal disease, kava use, malignancy, heart disease (rheumatic heart disease or congestive cardiac failure), chronic lung disease, immunosuppression, and other. The criteria for defining hazardous alcohol use, chronic lung disease, chronic renal disease, and septic shock were as previously published [7]. Of the 1005 melioidosis patients, 155 (15%) had no identified risk factors and these were additionally analyzed as a no-risk category.

Assigning a LPS Type to 1005 *Burkholderia pseudomallei* Strains Using Corresponding Whole-Genome Sequencing Data

The presence of LPS A, B, and B2 among the 1005 *B pseudomallei* genomes was determined using the Basic Local Alignment Search Tool (BLAST) [21]. LPS A (wbil to apaH

[location: 3196645–3215231 in K96243 NC_006350]), LPS B (BUC_3392 to apaH [location: 499502–535038 in *B pseudomallei* 579 NZ_ACCE01000003]), and LPS B2 (BURP840_LPSb01 to BURP840_LPSb21 [location: 241–26545 in *B pseudomallei* MSHR840] GU574442) were aligned against the in-house *B pseudomallei* database consisting of the 1005 strains using the nucleotide BLAST (BLASTn) parameter, and each genome was assigned an LPS type. The genomes were considered to match an LPS type if the percentage identity was >99%, the alignment length was 100%, and the E-value was 0. Genes were considered as absent if no BLAST hit was found or if the percentage identity, alignment length, and E-value did not meet the cutoff values listed above.

Correlation of In Silico LPS Genotype With Phenotype

Assigning an LPS serological phenotype by electrophoresis has previously been performed for 214 *B pseudomallei* strains with a Northern Territory origin [16]. Of these, 95 strains are included in this study, and for these we correlated the in silico-assigned LPS genotype with the prior published LPS phenotype.

Statistical Analysis

The required patient and strain/genome information were analyzed using Stata version 14.2 (StataCorp LP, College Station, TX). Bivariate analysis was performed using Pearson's χ^2 (frequency >5 in all cells) or Fishers exact tests (frequency <5 in all cells): $P < .05$ was considered significant. The Wilcoxon rank-sum test was used to compare age at diagnosis for LPS types A and B. Multivariate logistics regression was performed where significant bivariate P values were detected as previously described [7], to rule out confounding factors associated with LPS type status and clinical outcome.

RESULTS

LPS Phenotype Correlates With the LPS In Silico Genotype

For the 95 *B pseudomallei* strains in this study with a prior published electrophoretic phenotype [16], there was complete correlation with the in silico whole-genome sequence-determined genotype in 92 (97%) (73 LPS A, 19 LPS B, but no LPS B2). Three strains with an LPS A genotype were found by electrophoresis to have an alternative banding pattern that has been attributed to mutations in the O-antigen [16]. In addition, we performed genotyping of the 2 phenotypically LPS B2 strains from the prior paper (MSHR0446 and MSHR0840) [16] and showed concordance for both.

LPS Diversity and Associations Between LPS Types A and B With Clinical Parameters in Melioidosis Patients

LPS A was the predominant LPS type ($n = 876$; 87%) followed by LPS B ($n = 123$; 12%) and LPS B2 ($n = 6$; 1%). We next performed bivariate statistical analysis of LPS type A and B correlations with melioidosis clinical and epidemiological parameters.

Low LPS B2 numbers precluded statistical analysis; however, no epidemiological or clinical differences were evident compared with LPS A and B (Table 1). Bivariate statistical analysis of blood culture, septic shock, and mortality revealed no differential severity between patients with LPS types A and B (Table 1). Bivariate analysis did reveal a statistically significant association between LPS type B and presentation with osteomyelitis ($P = .049$) as well as several host factors including kava use ($P = .000$), history of an inoculation event ($P = .022$), and younger age ($P = .001$) (Table 1). Multivariate analysis suggested that these associations were likely explained by a strong geographical association between LPS type B and patient residence and infection in remote Northern Territory communities outside the urban capital of Darwin (data not shown).

DISCUSSION

Burkholderia pseudomallei is a facultative intracellular bacterium that can infect and invade most mammalian tissues and cell types. This broad cellular tropism gives *B*

pseudomallei its versatility and contributes to the diverse clinical presentations and outcomes observed in melioidosis patients [22]. *Burkholderia pseudomallei* is equipped with both ubiquitously and variably present virulence factors. Ubiquitous virulence factors include the cytotoxin *Burkholderia* lethal factor 1 [10], capsular polysaccharide I [11], type VI secretion system [9], the Bsa type III secretion system cluster 3 [12], and the disulphide bond proteins BpsDsbA and BpsDsbB [13]. One example of a variably present virulence factor is the *Burkholderia* intracellular motility protein A (BimA) variant *bimA_{Bm}*, which is present in 12% of north Australian strains and has been associated with neurological melioidosis in northern Australia [23]. Another important variably present virulence gene is the filamentous hemagglutinin 3 (*fhaB3*) gene, which has to date been found in all Thai strains but is absent in a minority of northern Australian strains [23]. *Burkholderia pseudomallei* lacking *fhaB3* are associated with cutaneous disease presentations and a lower propensity for bacteremic spread and death [23].

Table 1. Bivariate Clinical Associations With LPS Type A and B: The Frequency of LPS B2 for Each Clinical Association Is Listed

Primary Diagnosis	<i>lpsA</i> ^a	<i>lpsB</i> ^a	<i>P</i>	<i>lpsB2</i> ^b
Pneumonia	465 (53.1%)	60 (48.8%)	.371	2
Genitourinary presentation	105 (12.0%)	21 (17.1%)	.112	3
Blood culture positive, no focus	113 (12.9%)	10 (8.1%)	.132	0
Localized skin infection without sepsis	101 (11.5%)	15 (12.2%)	.829	1
Neurological presentation	14 (1.6%)	2 (1.6%)	.605 ^c	0
Soft tissue abscess	35 (4.0%)	6 (4.9%)	.644	0
Osteomyelitis	8 (0.9%)	4 (3.3%)	.049 ^c	0
Septic arthritis	21 (2.4%)	3 (2.4%)	.583 ^c	0
Other	14 (1.6%)	2 (1.6%)	1.000 ^c	0
Disease Severity Metrics				
Blood culture positive	498 (56.8%)	65 (52.8%)	.245	3
Septic shock	192 (21.9%)	22 (17.9%)	.307	3
Died from infection	108 (12.3%)	15 (12.2%)	.966	2
Risk Factors				
Inoculation event	177 (20.2%)	36 (39.3%)	.022	0
Hazardous alcohol use	354 (40.4%)	51 (41.5%)	.824	4
Diabetic	389 (44.4%)	54 (43.9%)	.916	4
Chronic renal disease	118 (13.5%)	9 (7.3%)	.055	1
Kava use	24 (2.7%)	13 (10.6%)	.000	0
Malignancy	86 (9.8%)	6 (4.9%)	.076	0
Rheumatic heart disease/congestive cardiac failure	78 (8.9%)	8 (6.5%)	.374	0
Chronic lung disease	238 (27.2%)	28 (22.8%)	.301	2
Immunosuppression	90 (10.3%)	8 (6.5%)	.188	0
Other	32 (3.7%)	3 (2.4%)	.493 ^c	1
No risk factors	134 (15.4%)	21 (17.0%)	.789	0
Gender (male)	546 (62.3%)	83 (67.5%)	.268	3
Indigenous Australian	455 (51.9%)	72 (58.5%)	.170	3
Median age (years)	50	44	.001	52

Abbreviations: LPS, lipopolysaccharide.

^aPercentages indicate the proportion of cases positive for a given primary diagnosis, disease severity metric, or risk factor according to *lpsA* or *lpsB*.

^bBivariate analysis not performed, $n = 6$; $n = 999$ for all bivariate tests.

^c*P* values calculated using the Fishers exact test.

The involvement of the LPS O-antigen moiety in *B pseudomallei* virulence was first described in the late 1990s [18]. Subsequently, diversity was shown to occur within *B pseudomallei* LPS [16], with serotypes A, B, and B2 described and regional differences in serotype prevalence noted [16, 24]. In vitro work with different LPS types suggested that diversity of the LPS O-antigen is associated with differential virulence [16, 17, 25]. Strains with LPS type A and B resisted serum killing and survived whereas LPS B2 strains were killed [16], demonstrating that LPS A and B strains have a potential survival advantage. This differential serum survival has also been noted in *Escherichia coli*, where certain O-antigen types provide a survival advantage in the presence of whole blood [26]. The lack of the LPS O-antigen has also been associated with increased susceptibility to macrophage killing, further demonstrating a possible role for the LPS O-antigen in intracellular survival [17]. Finally, LPS type B generates a significantly greater immune response compared with LPS type A, supporting that the different LPS types might cause different innate immune and adaptive responses among infected hosts [25]. The implications of these in vitro LPS studies is that different LPS types might be associated with different melioidosis disease outcomes in human infections, with ramifications for both biosecurity and vaccine development. To date, no studies have investigated whether the noted LPS diversity translates to clinical differences in disease presentations, severity, or outcomes in human melioidosis.

Our data confirm that LPS genotypes are unevenly distributed in the clinical *B pseudomallei* population, with LPS type A being dominant in northern Australia, as previously seen also in Thailand [16]. Bivariate analysis demonstrated that there is no signal for increased virulence in human melioidosis between LPS types, with LPS type A and B infections having similar rates of bacteremia, septic shock, and mortality. Although septic shock is triggered by the host responding to LPS [27], it is both the LPS O-antigen and lipid A component that are recognized during human infection, and both can determine the immunogenicity of LPS [17, 28–30]. Although this study does not support the involvement of the different LPS O-antigen types in conferring clinically significant differential virulence in human infection, *B pseudomallei* has also been shown to have subtle diversity in lipid A, which in itself may have various effects on host recognition [25, 31] and thus influence the progression to sepsis or other disease states. Further analysis of structural diversity of lipid A and potential associations with disease severity and outcomes are needed to rule out an association with differential virulence in human melioidosis.

The immune function of melioidosis patients plays a vital role in determining the propensity of infection to lead to clinical disease and determining the severity of disease and mortality [32]. Supporting this observation, 84% of melioidosis patients included in this study had a recognized risk factor for melioidosis, with diabetes being the most common (45% of

patients), similar to all other studies of melioidosis risk factors [33]. Both innate and acquired immunity are impacted by diabetes and the other recognized risk factors for melioidosis [34, 35]. Severe melioidosis disease and death are uncommon in healthy hosts, provided there is timely diagnosis, appropriate antibiotic therapy, and access to state-of-the-art intensive care support for patients with severe sepsis. The substantial decrease in overall mortality seen in the Darwin Prospective Melioidosis study, with no deaths in healthy hosts for over a decade, has led to the concept of melioidosis as an “opportunistic pathogen,” where host risk factors are of greater importance in determining disease severity and outcomes than differential virulence amongst *B pseudomallei* strains [36]. The lack of disease severity association with LPS genotype found in this study supports this concept.

An important limitation of the present study is that it only investigated the association of a single virulence mechanism with melioidosis clinical outcomes. In both the environment and in mammalian hosts, multiple virulence mechanisms of *B pseudomallei* work in concert, enabling *B pseudomallei* to invade host cells and cause disease through complex pathogenetic pathways. Microbial genome-wide association studies are now needed to elucidate whether combinations of virulence genes are associated with both disease presentations and with severity and outcomes. It is also documented that for rare strains of *B pseudomallei*, point mutations within LPS may alter phenotype without changing genotype [16, 25], and in this study we did not assess for such mutations. Finally, this study only included strains with an Australian origin, and studies investigating the impact of LPS diversity on virulence are needed for strains from other melioidosis-endemic regions.

CONCLUSIONS

In summary, the lack of influence of LPS genotype on clinical parameters in a large series of human melioidosis patients supports the primary role of host risk factors in determining disease severity and melioidosis outcomes.

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