Variable Virulence Genes in Clinical Isolates of *Burkholderia pseudomallei*: Impact on Disease Severity and Outcome in Melioidosis

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Abstract

Objectives To isolate *Burkholderia pseudomallei* from clinical specimens and study the association of virulence genes with clinical manifestations and outcome in patients with melioidosis.

Materials and Methods *Burkholderia pseudomallei* isolates obtained from melioidosis cases diagnosed during 2018 to 2021 were identified using VITEK 2 system and confirmed by polymerase chain reaction (PCR) targeting a Type III secretion system gene cluster. Multiplex PCR was performed to detect the genotypes of lipopolysaccharide (LPS) namely A, B, and B2, and singleplex PCR was performed to detect the presence of the *Burkholderia* intracellular motility gene (*BimA*) and filamentous hemagglutinin gene (*fhaB3*).

Statistical Analysis Chi-square/Fisher's exact tests were performed to study the association between various clinical manifestations and outcome and different virulence genes. The results were expressed as unadjusted odds ratios with 95% confidence intervals.

Results Sixty-seven isolates were available for characterization. $BimA_{Bm}$ and $BimA_{Bp}$ were observed among 82 and 18% of the isolates, respectively. Both sepsis and mortality were significantly associated with $BimA_{Bm}$. Majority of the isolates had *fhaB3* (97%). Most of the isolates showed the presence of LPS A gene (65.7%) followed by LPS B gene (6%), while LPS B2 was not detected. Nineteen isolates could not be assigned to any LPS genotypes.

Conclusion Among the virulence genes studied, only $BimA_{Bm}$ was significantly associated with sepsis and mortality. More than a quarter (28.3%) of the isolates could not be assigned to any LPS genotypes, hinting at a greater genetic diversity in our isolates.

Keywords
► *Burkholderia pseudomallei*
► melioidosis
► virulence genes

Introduction

Burkholderia pseudomallei is a motile nonspore forming, glucose nonfermenting opportunistic gram-negative organism that causes a fatal multifaceted disease, melioidosis.[1] Due to the absence of pathogen-specific clinical features, lack of suspicion and limited awareness among clinicians and microbiologists, this disease is underreported in India.

Melioidosis often manifests with varied clinical presentations that range from mild localized infection to fulminant sepsis.[2] The wide spectrum of disease could be attributed to the multiple virulence factors present in the bacteria that could possibly influence the disease severity.[3]

Three genotypes of lipopolysaccharide (LPS) (A, B, and B2), the *Burkholderia* intracellular motility gene (*BimA*), and the filamentous hemagglutinin gene (*fhaB3*) are some of the known virulence factors. Extensive studies on the frequencies of variable virulence factors present in *B. pseudomallei* and their associations with different clinical manifestations of melioidosis have been performed in Thailand and Australia where the disease is endemic, while there is only one such study from India.[2–5] Hence, this study aimed to isolate *B. pseudomallei* and document the association of virulence genes with clinical manifestations and outcome in patients with melioidosis.

Materials and Methods

This study was performed in a tertiary care institute in Southern India during the period, January 2018 to June 2021 after approval by the Institutional Ethics Committee (IEC) for Human Studies (JIP/IEC/2018/0230).

Isolation of *B. pseudomallei* from Patients Suspected to Have Melioidosis

All the specimens except blood were cultured on 5% sheep blood agar and MacConkey agar, whereas the BacT-Alert automated system (bioMérieux, Marcy l'Etoile, France) was used for blood samples. Isolates were identified by conventional biochemical tests and VITEK 2 system (bioMérieux) and confirmed by polymerase chain reaction (PCR) targeting a Type III secretion system gene cluster (TTS1), using previously published primers.[6]

Antibiotic Susceptibility Testing

In vitro antimicrobial susceptibility was performed using Kirby–Bauer disk diffusion method against meropenem (10 μg), imipenem (10 μg), ceftazidime (30 μg), doxycycline (30 μg), amoxicillin/clavulanic acid (30 μg), and trimethoprim–sulfamethoxazole (1.25/23.75 μg) and interpreted following protocols laid down by Mahidol University.[7] Isolates resistant to trimethoprim–sulfamethoxazole were retested by E-test.

Characterization of *B. pseudomallei* Isolates

Colony Morphotypes on Ashdown's Agar
All the isolates were plated on the Ashdown's agar to study their colony morphotypes. The plates were examined after

4 days of incubation at 37°C. The colony variations were identified as described previously, based on their color, size, translucency, wetness, circumference, and overall shape of the colony.[8]

Detection of Virulence Genes
Genomic DNA was extracted using Mericon DNA Bacteria Plus kit (Qiagen, Germany) according to the manufacturer's instructions. Multiplex PCR was performed to detect LPS genotype (A, B, and B2), whereas singleplex PCR was performed to detect the presence of the *BimA* gene variants (*BimA*$_{Bp}$ and *BimA*$_{Bm}$) and *fhaB3* using published primers and protocols with slight modifications (►**Supplementary Tables 1** and **2**, available in the online version).[2]

Statistical Analysis
All the categorical variables were summarized using frequency and percentages. Chi-square/Fisher's exact tests were performed to study the association between various clinical manifestations and outcome and different virulence genes. Unadjusted odds ratios (Unadj ORs) and their 95% confidence intervals (95% CIs) were estimated to assess the strength of association. A *p*-value less than 0.05 was defined as statistically significant. All the statistical analyses were performed using IBM SPSS Version 19.0.

Results

Of the 70 melioidosis cases diagnosed, only 67 isolates were characterized as one case was diagnosed by the presence of antigen in cerebrospinal fluid alone, while isolates from two other patients were lost due to contamination after confirming the identification. Isolates were provisionally identified based on the following characteristic features: motile gram-negative bacilli with bipolar staining, grayish white colonies with metallic sheen on 5% sheep blood agar and wrinkled pink colored colonies on MacConkey agar, oxidase positive, oxidative utilization of lactose, arginine dihydrolase positive, and lysine decarboxylase negative. There was no discrepancy in the results of the identification methods employed. Among the 69 isolates, 63 were susceptible to meropenem, imipenem, ceftazidime, doxycycline, amoxicillin/clavulanic acid and 6 isolates were resistant to trimethoprim–sulfamethoxazole by Kirby–Bauer disk diffusion method. These six isolates were found to be sensitive by E-test.

Clinical Features
Of the 67 melioidosis cases, there were 33 (49.3%) patients with sepsis, 18 (26.9%) with pneumonia, 22 (32.8%) with superficial abscesses, 23 (34.3%) with deep organ abscesses, 14 (20.9%) with septic arthritis, and 6 (9%) with neurological melioidosis. Overall mortality was 35 (52.2%).

Characterization of *B. pseudomallei* Isolates
Among the 7 morphotypes described, 27 (40.3%) isolates belonged to type I (►**Fig. 1**). None of the isolates resembled the types III and V. A higher proportion of types II and IV
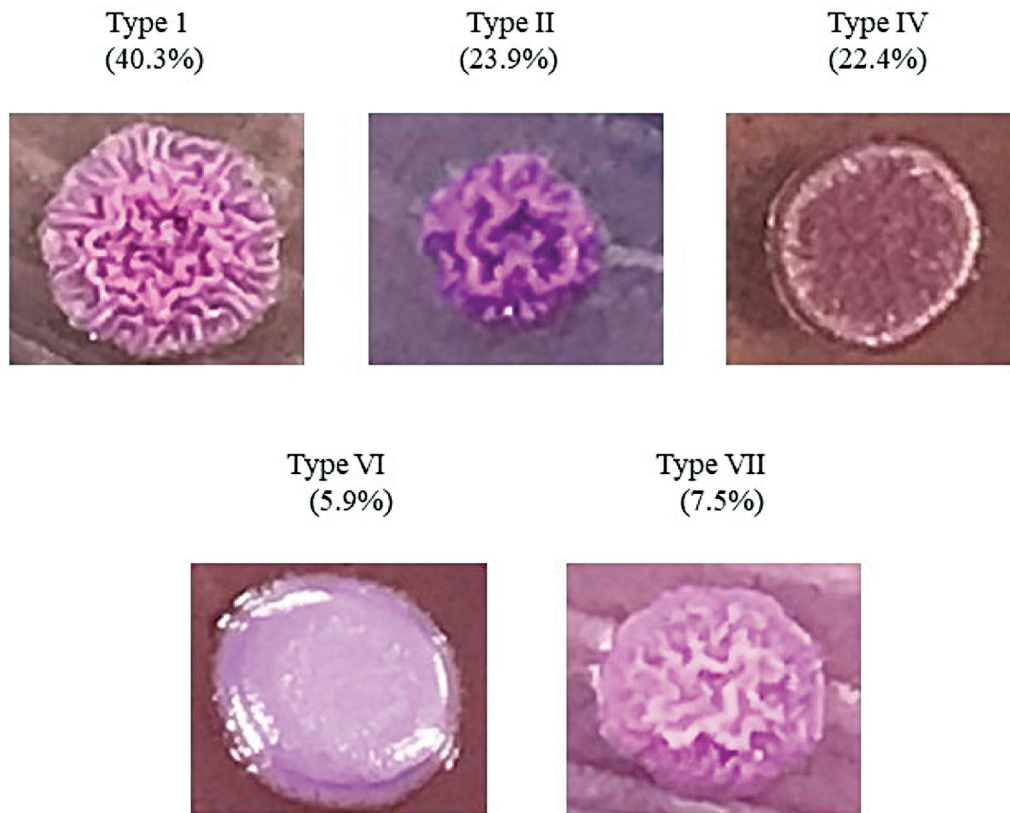
Type 1
(40.3%)

Type II
(23.9%)

Type IV
(22.4%)

Type VI
(5.9%)

Type VII
(7.5%)

Fig. 1 Colony morphotypes of *B. pseudomallei* isolates.

morphotypes (31% each) were seen in patients with a fatal outcome (►**Table1**).

Virulence Genes and Their Association with Clinical Presentations and Outcome

Most of the isolates showed the presence of LPS A gene (44, 65.7%) followed by LPS B gene (4, 6%), while LPS B2 was not detected (►**Table 2**). Nineteen isolates (28.3%) could not be assigned to any LPS genotype. Majority of the isolates had *fhaB3* gene (97%). Among the *BimA* gene variants, $BimA_{Bm}$ and

$BimA_{Bp}$ were observed among 82 and 18% of the isolates, respectively. None of the LPS genotypes had a significant association with the clinical presentations. However, between the *BimA* gene variants, $BimA_{Bm}$ was significantly associated with both sepsis and mortality. The odds of having sepsis in patients with $BimA_{Bm}$ was 6.46-fold higher than those with $BimA_{Bp}$ (Unadj OR = 6.46 [95% CI: 1.29, 32.28], $p = 0.01$), whereas the likelihood of fatal outcome was 4.17-fold higher in $BimA_{Bm}$ (Unadj OR = 4.17 [95% CI: 1.02, 17.13], $p = 0.04$) when compared with $BimA_{Bp}$ (►**Table 2** and ►**Fig. 2**).

Table 1 Distribution of colony morphotypes of *B. pseudomallei* in melioidosis patients

Colony morphotypes (n = 67)	Bacteremia (n = 47)	Pneumonia (n = 18)	Septic arthritis (n = 14)	Neuromelioidosis (n = 6)	Sepsis (n = 33)	Superficial abscess (n = 22)	DOA (n = 23)	Mortality (n = 35)
Type I 27 (40.3)	17 (36.2)	4 (22.2)	7 (50)	3 (50)	10 (30.3)	10 (45.5)	11 (47.8)	9 (25.7)
Type II 16 (23.9)	13 (27.7)	7 (38.9)	3 (21.4)	2 (33.3)	10 (30.3)	4 (18.2)	6 (26.1)	11 (31.4)
Type IV 15 (22.4)	12 (25.5)	6 (33.3)	3 (21.4)	1 (16.7)	10 (30.3)	3 (13.6)	3 (13.0)	11 (31.4)
Type VI 4 (5.9)	3 (6.4)	1 (5.6)	0	0	2 (6.1)	3 (13.6)	1 (4.3)	2 (5.7)
Type VII 5 (7.5)	2 (4.3)	0	1 (7.1)	0	1 (3.0)	2 (9.1)	2 (8.7)	2 (5.7)

Abbreviation: DOA, deep organ abscess.

Table 2 Association of virulence genes of *B. pseudomallei* isolates with clinical presentations and mortality

Variables (n = 67)	Bacteremia (n = 47)	Pneumonia (n = 18)	Septic arthritis (n = 14)	Neuromelioidosis (n = 6)	Sepsis (n = 33)	Superficial abscess (n = 22)	DOA (n = 23)	Mortality (n = 35)
LPS[a]								
A (44)	30 (90.9)	12 (92.3)	9 (90.0)	3 (100.0)	18 (90.0)	18 (94.7)	16 (94.1)	19 (86.4)
B (4)	3 (9.1)	1 (7.7)	1 (10.0)		2 (10.0)	1 (5.3)	1 (5.9)	3 (13.6)
p-Value	1.00	1.00	1.00	0	1.00	1.00	1.00	0.32
OR	0.71	1.13	0.77	1.00	0.69	2.08	1.71	0.25
95% CI	0.07, 7.49	0.11, 11.9	0.07, 8.3		0.09, 5.38	0.20, 21.6	0.16, 17.8	0.02, 2.63
fhaB3								
Yes (65)	45 (95.7)	17 (94.4)	13 (92.9)	6 (100.0)	31 (93.9)	22 (100.0)	23 (100.0)	33 (94.3)
No (2)	2 (4.3)	1 (5.6)	1 (7.1)	0 (0.0)	2 (6.1)	0 (0.0)	0 (0.0)	2 (5.7)
p-Value	1.00	0.47	0.38	1.00	0.24	1.00	0.54	0.49
OR		0.35	0.25					
95% CI		0.02, 5.98	0.02, 4.27					
BimA variants								
BimA$_{Bm}$ (55)	40 (85.1)	17 (94.4)	12 (85.7)	5 (83.3)	31 (93.9)	18 (81.8)	17 (73.9)	32 (91.4)
BimA$_{Bp}$ (12)	7 (14.9)	1 (5.6)	2 (14.3)	1 (16.7)	2 (6.1)	4 (18.2)	6 (26.1)	3 (8.6)
p-Value	0.32	0.16	1.00	1.00	0.01[b]	1.00	0.31	0.04[b]
OR	1.91	4.92	1.40	1.10	6.46	0.97	0.45	4.17
95% CI	0.52, 6.93	0.59, 41.22	0.27, 7.25	0.12, 10.38	1.29, 32.28	0.26, 3.66	0.13, 1.59	1.02, 17.13

Abbreviations: BimA, *Burkholderia* intracellular motility gene; CI, confidence interval; DOA, deep organ abscess; *fhaB3*, filamentous hemagglutinin gene; LPS, lipopolysaccharide; OR, odds ratio.
[a]Nineteen isolates could not be assigned to any LPS genotypes and were excluded from the analysis.
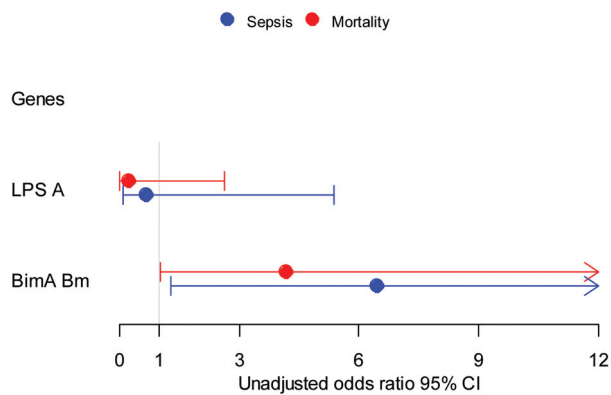[b]Chi-square test.

Fig. 2 Association of virulence genes (LPS and *BimA*) with sepsis and mortality. CI, confidence interval; LPS, lipopolysaccharide.

Discussion

Melioidosis is diagnosed by isolation of *B. pseudomallei* from any clinical specimen since it is never a part of normal flora.[9] The nonspecific clinical manifestations of melioidosis make the diagnosis difficult. Therefore, culture remains the mainstay of diagnosis.

The use of multiple methods is recommended due to the misidentification of *B. pseudomallei* colonies with standard biochemical tests.[10] Therefore, in the present study, all the isolates identified by biochemical tests initially were confirmed using VITEK 2 system and further confirmed by PCR, targeting a TTS1.[6] TTS1 is only present in *B. pseudomallei* and not in the other closely related species such as *B. mallei* or *B. thailandensis*.[11] Hence, PCR helps in additional confirmation as it is species specific and can be reported within 6 to 8 hours.[6]

Burkholderia pseudomallei exhibits variation in the colony morphology ranging from typically wrinkled to smooth colonies. Lack of awareness of these colony variants among the microbiologists can lead to underdiagnosis of melioidosis in nonendemic regions. Seven morphotypes (I–VII) have been described based on the colony variations in Ashdown's agar.[8] In a study by Chantratita et al, 75.1% of the *B. pseudomallei* strains were type I and the remaining 24.9% of strains belonged to the other types (II–VII). Similarly, in the present study, majority of the isolates belonged to type I (40.3%, ►**Table 1**). However, types (III and V) were not found among the study isolates. Type I is the parental morphotype observed during the clinical infection that gives rise to the other six morphotypes in vivo.[8] In the present study, among the patients who expired types II and IV were the most common morphotypes observed.

The extensive cellular tropism in facultative intracellular *B. pseudomallei* accounts for the protean clinical presentations and outcomes.[5] Both universal and variable virulence factors in *B. pseudomallei* play a central role in disease pathogenesis. Capsular polysaccharide I, Bsa TTS1, type VI secretion system, cytotoxin *Burkholderia* lethal factor 1, and the disulfide bond proteins (BpsDsbA and BpsDsbB) are a few universal and the *Burkholderia* intracellular motility gene (*BimA*) and the filamentous hemagglutinin gene (*fhaB3*) are variably present virulence factors.[2,5]

Another virulence factor is LPS with three serotypes A, B, and B2. LPS diversity associated with differential virulence and regional differences in type prevalence has been reported previously.[12,13] In vitro work demonstrated that LPS genotypes A and B have a survival advantage and resist serum killing unlike LPSB2 strains.[12] Further, LPS mutant lacking O-antigen moiety was susceptible to macrophage killing which supports the importance of LPS O-antigen in intracellular survival.[12] Compared with LPS genotype A, type B elicited a greater immune response indicating substantial differences in the host response to LPS.[14] These in vitro findings imply that LPS diversity might be linked with various clinical presentations and outcome.

The present study confirms that LPS genotypes are disproportionately distributed among the clinical isolates. LPS genotype A was the most predominant genotype found in Southeast Asia (97.7%), Australia (85.3%), Myanmar (63%), and Malaysia (96.4%) which is similar to the findings from the present study where majority of the patients (65.7%) were infected with LPS genotype A.[12,15,16] Frequency of LPS genotype B in the present study was 6% compared with 2.3% in Southeast Asia, 12 to 13.8% in Australia, 37% in Myanmar, and 3.6% in Malaysia.[5,12,15,16] This is in contrast to the findings from Manipal, where LPS genotype B (74%) was reported to be prevalent.[2]

The presence of LPS genotype B2 is rare compared with the other two genotypes (A and B). LPS genotype B2 was found in 1 to 8.3% of isolates from Australia, 5.5% from Manipal, while none of the isolates from Myanmar and Malaysia possessed this genotype.[2,5,12,15,16] Failure to find LPS genotype B2 in the present study and the studies from Myanmar and Malaysia could be attributed to the small sample size. The most intriguing finding in the present study was failure to detect the LPS genotype in 19 (28.3%) isolates. Similar findings were reported by Webb et al, and they attributed it to some mutations in the O-antigen, leading to an alternate banding pattern. These variants were later confirmed to be of the LPS genotype A by genome sequencing. While this was a minor number (3/95), in the present study, 28.3% were not assigned to any of the LPS genotypes.[5] This shows a possible greater genetic diversity in the O-antigen.

However, neither of the LPS genotypes had significant association with the clinical presentations or outcome. This was similar to the studies from Australia and Manipal.[2,5] Therefore, the diversity in LPS genotype may not explain the differences in clinical presentations or outcomes, instead host risk factors may play a more critical role.

Filamentous hemagglutinin 3 (*fhaB3*) gene is another virulence candidate present in *B. pseudomallei* that encodes for filamentous hemagglutinin, a surface protein. The multiple functions associated with the antimacrophage factor *fhaB3* are adhesion to the host epithelial cells, actin-based membrane protrusions, and formation of multinucleated giant cells.[3] *fhaB3* was present in 100% of the isolates from Thailand, 83% from Australia, and 69% from Myanmar.[3,15,17]

The presence of *fhaB3* was reported in 95% of *B. pseudomallei* isolates from Manipal.[2] In the current study, 97% of the isolates possessed *fhaB3* gene. In line with the Manipal study, the gene had no significant association with any form of the disease.[2]

BimA$_{Bm}$ and *BimA$_{Bp}$* are the variants of *Burkholderia* intracellular motility factor A gene (*BimA*), a protein that is required for actin tail formation and actin-based motility. Both the variants were reported among the isolates from Australia, whereas only *BimA$_{Bp}$* was reported from Southeast Asian countries.[3] Shaw et al from Manipal reported *BimA$_{Bp}$* in 95.4% and *BimA$_{Bm}$* in 4.5% of isolates. Moreover, *BimA$_{Bm}$* has been associated with neuromelioidosis in Australia and Manipal.[2,3] Such an association was not found in the present study which could be due to the low number of neuromelioidosis cases. Unlike the Manipal study, *BimA$_{Bm}$* variant was predominant and was found to be significantly associated with sepsis and mortality among our patients. Although *BimA$_{Bm}$* significantly increased the odds of either sepsis or mortality, it was not associated with mortality due to sepsis. This implies that host factors are responsible for mortality in sepsis rather than the presence of *BimA$_{Bm}$*.

Conclusion

Compared with the *B. pseudomallei* isolates reported worldwide, this study highlights the divergence of Indian strains in context to LPS genotype as 28.3% of the isolates could not be assigned to any LPS genotypes, hinting at a greater genetic diversity in our isolates. Among the virulence genes identified, *BimA$_{Bm}$* was significantly associated with sepsis and mortality.

Conflict of Interest
None declared.

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