Sequence Polymorphism and PCR-Restriction Fragment Length Polymorphism Analysis of the Flagellin Gene of *Burkholderia pseudomallei*[▽]

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Four flagellin allelic types (I to IV) of *Burkholderia pseudomallei* were identified based on their sequence variation and restriction fragment length polymorphism (RFLP) analysis of the amplified flagellin gene. Flagellin allelic type I was the most predominantly (75.0%) found among the 100 clinical isolates of *B. pseudomallei* investigated in this study.

Melioidosis is a serious infection caused by the soil saprophyte *Burkholderia pseudomallei*, and it is capable of becoming a dangerous pathogen particularly in individuals with one or more preexisting conditions associated with an altered immune response. This infection, commonly reported from southeast Asia and northern Australia, is being recognized increasingly around the world (6, 8, 13). The flagellin gene has been used to differentiate or subtype many bacterial strains (15). PCR amplification combined with restriction fragment length polymorphism (RFLP) or sequence analysis has been used to investigate the variation of the *B. pseudomallei* flagellin gene (*fliC*) (9, 10, 12, 14). However, no sequence variation has been reported so far, probably due to the small number of isolates investigated in these studies. Recently, *B. pseudomallei* flagellin protein has been recognized as a protective immunogen and a potential vaccine target for melioidosis (2, 4, 7, 16). The flagellin protein also has been found to be an efficient antigen for serodiagnosis (5). As antigenic variation may be generated by different flagellin types, knowledge of a particular flagellin type of *B. pseudomallei* in the regions in which it is endemic may be useful for designing effective diagnostic reagents and vaccine.

This study was undertaken first to determine the sequence polymorphism of the *fliC* gene of a collection of genetically diverse *B. pseudomallei* isolates (as indicated by different randomly amplified polymorphism DNA [RAPD] profiles). A PCR-RFLP method then was developed to facilitate the recognition of *B. pseudomallei* flagellin allelic types. The distribution of the flagellin allelic types of 100 Malaysian clinical isolates then was determined.

The PCR primers used for *fliC* amplification were BC6E (5'-ACCAACAGCCTGCAGCGTATC-3') and BCR14 (5'-TATTGCAGGAGCTTCAGCAC-3') (14). The amplification mixture (total volume of 50 μ l) contained 5 μ l of 10 $\times$ *Taq* buffer with KCl; 2 mM MgCl₂; 0.2 mM (each) dATP, dCTP, dGTP, and dTTP; 0.5 μ M primers; 2.5 U *Taq* DNA polymerase (MBI Fermentas); and 2.5 μ l of purified bacterial DNA.

Amplification was performed with denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1.5 min, with a final extension at 72°C for 10 min. The amplicons were purified and sequenced using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, CA) using primers BC6E and BCR14. *B. pseudomallei* ATCC 23343 and *Burkholderia thailandensis* ATCC 700388 were included in this investigation.

A total of 730 nucleotides (corresponding to bp 342 to 1072 of the variable region of *fliC* of *B. pseudomallei* ATCC 23343) were determined for 17 RAPD types of *B. pseudomallei*. Sequence analysis demonstrated four flagellin allelic types (I to IV) (Fig. 1). The *fliC* gene sequence of *B. thailandensis* greatly varied from that of *B. pseudomallei*, with 76 nucleotide differences and a deletion of 15 nucleotides. The nucleotide sequences of flagellin allelic type I were 100% identical to the *fliC* of *B. pseudomallei* reference strain ATCC 23343 as well as of strains 1106a, 668, 1710b, and E503 in GenBank. The flagellin allelic type II showed only one nucleotide difference (C instead of G at position 456) compared to the sequences of flagellin allelic type I, but it matched exactly with the *fliC* gene of *B. pseudomallei* K9623 and *B. mallei* NCTC 10247, 10229, SAVP1, and ATCC 23344.

The flagellin allelic types III and IV identified in this study are considered new sequence variants for *B. pseudomallei*, as identical sequences have not been reported in the public sequence databases. Flagellin allelic type III demonstrated two nucleotide differences (T instead of C at position 377 and C instead of G at position 456) from the sequence of flagellin allelic type I. Flagellin allelic type IV showed 12 nucleotide differences from allelic type I (Fig. 1). The flagellin allelic type IV in this study show 98% sequence similarity to the flagellin sequences of *B. pseudomallei* and *B. mallei* but 89% to that of *B. thailandensis*. Four sequence entries (AJ617736, AJ617737, AJ617738, and AJ617739) in the GenBank database had partial sequence identities (only 58% sequence coverage) to the flagellin allelic type IV identified in this study. These isolates had been detected with different melting temperatures by real-time PCR assays (11). While the single-nucleotide substitution for flagellin allelic type II did not result in an amino acid change, one nonsynonymous change (valine instead of alanine) was noted for flagellin allelic type III, whereas five nonsynony-

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FIG. 1. Sequence alignment of the variable region of the flagellin (*fliC*) genes of four allelic types of *B. pseudomallei*. The reference strains of *B. pseudomallei* ATCC 23343 and E503, *B. mallei* NCTC 10247, and *B. thailandensis* ATCC 700388 were included for comparison. Identical nucleotides are indicated by a dot, whereas deletions are indicated by a minus.

FIG. 2. Restriction profiles of four flagellin allelic types of *B. pseudomallei* isolates. Lanes: M, *pBR322*/HaeIII DNA marker; 1, flagellin allelic type I; 2, flagellin allelic type II; 3, flagellin allelic type III; 4, flagellin allelic type IV; 5, *B. thailandensis* ATCC 700388; 6, *B. pseudomallei* NCTC 13178; 7, *B. pseudomallei* ATCC 23343.

mous changes (two alanines instead of serines, one serine instead of threonine, one alanine instead of threonine, and one threonine instead of serine) were observed for flagellin allelic type IV.

Restriction analyses of amplified flagellin PCR products (10 μ l) were performed using 5 U each of MspI (5'CCGG3') and AsuI (5'GGNCC3') (MBI Fermentas) incubated overnight at 37°C. Four restriction profiles (Fig. 2) were generated for *B. pseudomallei* corresponding to the flagellin sequence analysis. The PCR-RFLP approach also was able to differentiate *B. thailandensis* from *B. pseudomallei* (Fig. 2). Of the 100 clinical isolates of *B. pseudomallei* investigated in this study, flagellin allelic type I was the most predominant (75.0%), followed by allelic type II (20.0%). Flagellin allelic types III and IV were identified in three (3.0%) and two (2.0%) clinical isolates, respectively (Table 1). No major difference was found in the distribution of flagellin allelic types for isolates obtained from blood cultures or other clinical specimens (Table 1).

Due to the lack of comprehensive patient follow-up data, we

are not able to comment on the clinical presentation and outcome of the patients infected by *B. pseudomallei* of different flagellin allelic types. Our preliminary study showed that the motility of the isolates was not affected by the allelic types. It has yet to be investigated whether minor amino acid changes observed for flagellin types III and IV of *B. pseudomallei* will have some effect on the expression and antigenicity of flagellin. In some bacteria, for example, the switch from alanine to serine or alanine to threonine in the central region of flagellin may cause posttranslational modifications that are responsible for the changes in the antigenicity of flagellin protein (1, 3).

Nucleotide sequence accession numbers. The sequences for *B. pseudomallei* flagellin allelic types I to IV were deposited in GenBank under accession numbers HM003221 to HM003224.

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TABLE 1. Distribution of flagellin allelic types in clinical isolates of *B. pseudomallei* by PCR-RFLP analysis

Flagellin allelic type	No. (%) of isolates		Total
	Blood culture	Other clinical specimen	
I	35 (70.0)	40 (80.0)	75 (75.0)
II	11 (22.0)	9 (18.0)	20 (20.0)
III	2 (4.0)	1 (2.0)	3 (3.0)
IV	2 (4.0)	0 (0.0)	2 (2.0)
Total	50 (100)	50 (100)	100 (100.0)