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Molecular Investigation of Virulence Determinants between a Virulent Clinical Strain and an Attenuated Strain of *Burkholderia pseudomallei*

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Key Words
Burkholderia pseudomallei · Subtractive hybridization · Virulence determinants

Abstract
Burkholderia pseudomallei is the causative agent of melioidosis. We initiated this investigation with a virulent and an attenuated strain of *B. pseudomallei*. Pulsed-field gel electrophoresis was carried out initially for macrogenomic comparison of both strains of *B. pseudomallei*. However, the pulsotypes obtained were identical and therefore we applied a subtractive hybridization technique to distinguish and determine the possible differences between the two strains. Six virulence strain-specific DNA fragments were obtained and the encoding homolog proteins were identified as a xenobiotic-responsive element family of transcriptional regulator, a hypothetical protein, an unknown protein, a plasmid recombination enzyme, a regulatory protein and a putative hemolysin activator protein. A combination of at least three of these determinants was identified in 45 clinical isolates when screening was carried out with self-designed multiplex PCR targeting the six putative virulent determinants. Our data demonstrated that different combinations of the six putative virulence genes were present in the clinical isolates indicating their probable role in the pathogenesis of *B. pseudomallei* infections.

Burkholderia pseudomallei, a Gram-negative bacilli, is an environmental saprophyte that possesses a remarkable capacity to infect humans and animals, causes a potentially serious infection known as melioidosis which is an important cause of sepsis in the tropics and is endemic in South-East Asia and Northern Australia. This infection has the potential for prolonged latency periods with recrudescence into acute and fulminating fatal infections [Puthucheary, 2009]. The potential severity of acute melioidosis indicates that the organism possesses potent virulence factors. Yet after many decades of research, there is a paucity of knowledge in this area compared to other Gram-negative bacteria and only a limited number of virulence factors have been elucidated so far. Some of these studies have been concerned with the role of exoproducts, such as protease, lipase, phospholipase C, quorum sensing, biofilm formation, type III secretion system and capsular polysaccharide [Wierasinga et al., 2006].

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The identification and characterization of virulence factors in *B. pseudomallei* has been carried out using various high-powered techniques such as subtractive hybridization (SH) which enables differentiation and molecular amplification of DNA fragments that are present in one bacterial genome (the tester strain) but absent in another (the driver strain). In addition, differences in relative abundance of transcripts are highlighted as well as genetic differences between species. Past published reports have focused on genomic differences between the virulent *B. pseudomallei* and a closely related non-infectious (avirulent) family member *Burkholderia thailandensis* [Reckseidler et al., 2001; Yu et al., 2006].

In this study, we have used a virulent clinical isolate of *B. pseudomallei (v)* and an attenuated strain of *B. pseudomallei (av)*, in a PCR-based SH technique to detect and characterize the presence of potential virulence determinants and their encoded genes. The virulent strain of *B. pseudomallei (v)* was isolated from the blood culture of a 44-year-old male diabetic in 1994, who subsequently succumbed to the infection. This virulent strain was used to prepare antigen for serological tests in melioidosis-positive patients. Overtime, in 2003, the freshly prepared antigen failed to give a positive reaction and the organism also did not agglutinate with our in-house polyvalent antiserum. When this live organism was injected into Balb/C mice that were 6–7 weeks old, the rate of survival, in days, was at least two times longer compared to a virulent strain (table 1). Henceforth this strain is referred to as the attenuated *B. pseudomallei (av)* strain. Phenotypically the virulent and attenuated strains of *B. pseudomallei* were identical by routine laboratory biochemical tests as well as by the API 20NE (bioMérieux, France) test.

Forty-five clinical isolates of *B. pseudomallei* were obtained from our culture collection. These had been isolated from patients in hospitals throughout Malaysia, from 1990 to 2008 and stored in glycerol at –80°C. Twenty-four were from blood cultures and 21 were non-blood isolates. The age of the patients ranged from 8 to 68 years; 31 were males and 14 were females. Animal ethics (Permit No. MIC/01/08/02/SDP(R)) and human Ethics approval (Permit No. 260.1) for this study had been obtained from the University of Malaya and the University of Malaya Medical Center (UMMC) review board, respectively.

The bacterial strains were grown in Luria-Bertani broth and genomic DNA was extracted using i-genomic CTB DNA extraction mini kit (iNtRON Biotechnology). Two milliliters of cultured bacterial cells were centrifuged and lysed for 30 min in lysis buffer at 65°C. After addition of binding buffer and 80% (v/v) ethanol, the mixture was transferred to a column and spun. The flow-through was discarded and the spin column was washed with buffer. The genomic DNA was then eluted out of the spin column with elution buffer.

Table 1. Mean of days of survival of mice challenged with *B. pseudomallei*

Balb/C	Bacterial strain	Survival rate, days			p value
		challenge dose, CFU/ml			
		10	10^2	10^3	
Male	virulent *(v)*	27	21	10	6.9×10^{-8}
	avirulent *(av)*	68	68	68	
Female	virulent *(v)*	32	23	12	3×10^{-7}
	avirulent *(av)*	69	67	69	

Mean number of survival days calculated from each group of 3 mice and statistical significance determined by Student's t test.

Pulsed-field gel electrophoresis (PFGE) was carried out according to previously published protocols [Chua et al., 2010; Chua et al., 2011; See et al., 2009]. Briefly, *B. pseudomallei* genomic DNA was used to prepare 1.6% (w/v) agarose plugs [Chua et al., 2009]. These plugs containing genomic DNA were digested overnight with *Spe*I enzyme (New England BioLabs, USA) for brief profiling comparison study. The DNA fragments were separated by electrophoresis (CHEF DR II; Bio-Rad Laboratories) at 14°C for 28 h at 200 V with pulse times ramped linearly from 5 to 65 s and the DNA fingerprints recorded.

SH was carried out between the virulent *(v)* (tester) and the attenuated *(av)* (driver) strains of *B. pseudomallei* in order to isolate DNA sequences encoding for possible virulence determinants unique to *B. pseudomallei (v)* strain as described previously [DeShazer, 2004]. The protocol of the A PCR-Select™ Bacterial Genome Subtraction Kit (Clontech, USA) was followed and the tester-specific *(v)* DNA fragments were ligated into a pGEM®-T Vector System containing T7 and SP6 sequencing priming sites (Promega). The ligated products were then transformed into One Shot® TOP10 competent cells (Invitrogen, USA). The transformation culture was spread on LB/ampicillin/IPTG/X-Gal plates and the white colonies carrying the putative plasmids with the tester-specific *(v)* DNA fragments were selected to undergo plasmid extraction using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, USA). The extracted plasmids were sequenced with T7 and SP6 primers to obtain information of genes pres-

Table 2. Primer sequences of the six putative virulence determinants

No.	Sequence (5′ to 3′)	Amplicon, bp	Target
1	GCTGGAATTCGTGGAATG CAAAGCCGATAGAATGGGAA	466	fragment 1
2	ACTTCGACCATCAGACCG GTTGCGAATGCGATGAC	440	fragment 2
3	ACTTTCGAATGCCTTCAAC GCTCGACTGACTCTTCATTC	364	fragment 3
4	TTACGCAAGCCAAGTCAATG AAGCACCTTTCCCTGGAACT	219	fragment 4
5	GGTCAGACGTGAAATAATCG GCCTGTTTGGAATAATGC	293	fragment 5
6	AAGGGAGGCAATCGACAGTA AATCCAGACCCATTCCG	114	fragment 6

Table 3. BlastX results for strain-specific DNA fragments present in *B. pseudomallei (v)*

DNA fragment	Size, bp	Identity, %	Protein information
1	900	97	transcriptional regulator, XRE family protein (*B. pseudomallei* B7210)
2	520	97	hypothetical protein (*B. pseudomallei* 1710a)
3	490	99	unknown protein (*B. pseudomallei* 1026b)
4	440	95	plasmid recombination enzyme (*B. multivorans* CGD1)
5	400	100	regulatory protein (*B. pseudomallei* 1106a)
6	220	100	putative hemolysin activator protein (*B. pseudomallei* 1710a)

ent in the tester *(v)* but absent in the driver *(av)* strain. Protein homology analysis of the acquired sequences was performed using the BlastX program at NCBI in search of *Burkholderia* proteins identity determination and data mining through PATRIC database [Gillespie et al., 2011].

Following the sequencing of the six fragments, six pairs of specific primers targeting and corresponding to the six potential virulence determinants were designed. These primer sequences are listed in table 2. Two sets of multiplex PCRs were developed to enable rapid screening of the 45 clinical isolates of *B. pseudomallei* for the presence of any of the six virulence determinants. The first set consisted sequences corresponding to fragments F2, F3 and F4 and the second set to F1, F5 and F6. Further checking of primer specificity was carried out using in silico PCR as previously described [Teh et al., 2010; Thong et al., 2011]. Two separate multiplex PCRs were carried out on the *B. pseudomallei (v)* clinical strain, attenuated *B. pseudomallei (av)* and the 45 clinical isolates of *B. pseudomallei.* PCR was performed in a final volume of 15 μl containing 100 ng of DNA template, 1× of buffer, 0.5 μM of each forward and reverse primers, 200 μM of dNTP mix, 1.5 mM of $MgCl_2$, 1 U of *Taq* DNA polymerase (Fermentas, USA) and an appropriate volume of distilled water. Cycling parameters consisted of denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 59°C for 30 s, 72°C for 60 s and a final extension at 72°C for 5 min. The PCR products were then electrophoresed on a 1.0% (w/v) agarose gel and stained with ethidium bromide.

PFGE analysis carried out on both the *(v)* and the *(av)* strains of *B. pseudomallei* resulted in similar pulsotypes (fig. 1), indicating that these two strains were indistinguishable in terms of macrogenomic features. Hence, SH was carried out between the virulent *(v)* (tester) and the

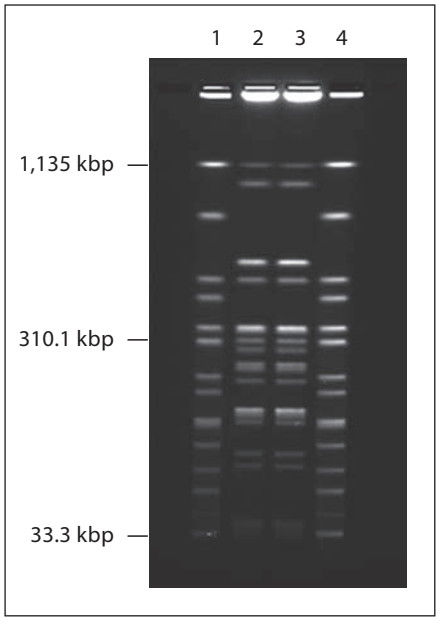

Fig. 1. PFGE pulsotypes of virulent *(v)* and attenuated *(av)* strain of *B. pseudomallei*. Lanes 1 and 4: *Xba*I-digested DNA of S*almonella* Braenderup (H9812: reference standard), lane 2: *Spe*I-digested DNA of virulent *B. pseudomallei (v)*, lane 3: *Spe*I-digested DNA of *(av)* strain of *B. pseudomallei*.

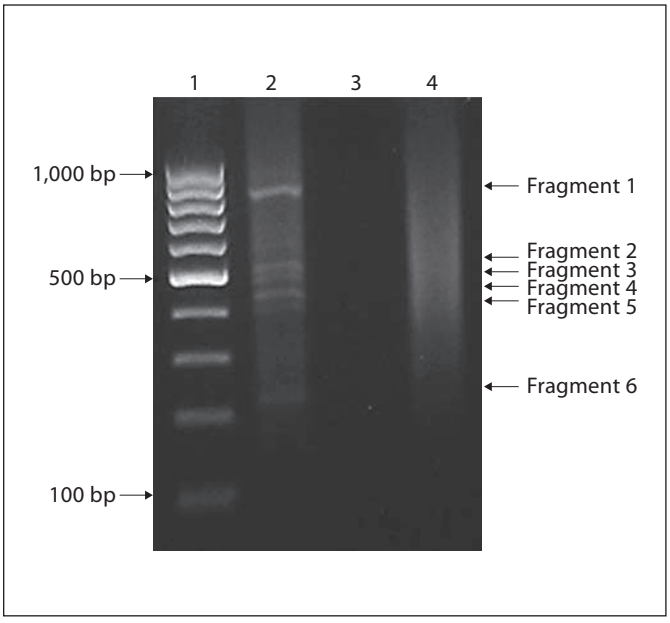

Fig. 2. SH of virulent *(v)* and attenuated *(av) B. pseudomallei*. Lane 1: 100 bp DNA ladder; lane 2: virulent *B. pseudomallei (v)* hybridized with attenuated *B. pseudomallei (av)*; lane 3: *B. pseudomallei (av)* hybridized with virulent *B. pseudomallei (v)*; lane 4: control (unsubtracted genomic DNA from virulent *B. pseudomallei (v)*).

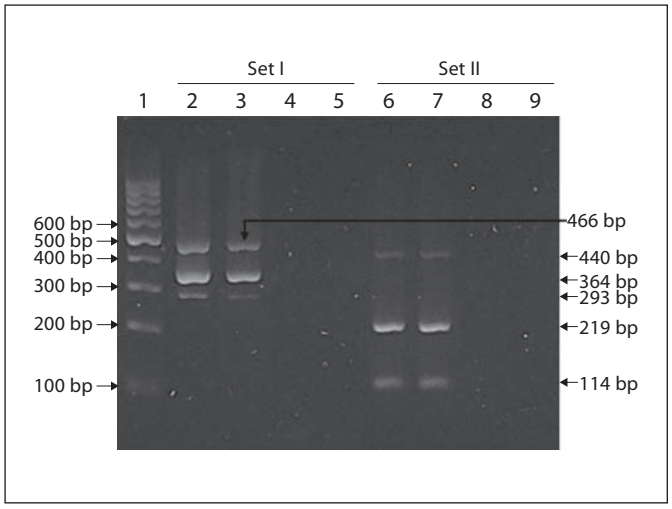

Fig. 3. Multiplex PCR results (Set I: 466 bp, 364 bp, 293 bp; Set II: 440 bp, 219 bp, 114 bp) of the 6 representative DNA fragments. Lane 1: 100 bp DNA standard; lanes 2 and 6: clinical isolate S4; lanes 3 and 7: control virulent strain; lanes 4 and 8: control attenuated strain; lanes 5 and 9: DNA blank.

attenuated *(av)* (driver) strains of *B. pseudomallei* which revealed six strain-specific DNA fragments that were unique to the virulent tester strain of *B. pseudomallei (v)* (fig. 2, lane 2), whereas these DNA fragments were not seen in the attenuated driver *(av)* strain (fig. 2, lane 3). The fragments in the tester *(v)* ranged from 220 to 900 bp in length and all the six fragments were subsequently sequenced.

The NCBI BlastX search results for the subtracted DNA fragments against *B. pseudomallei* proteins are summarized in table 3. The results revealed that only four of the six sequences had homology to the predicted proteins present in the GenBank database. These were identified as a xenobiotic-responsive element (XRE) family of transcriptional regulator, a plasmid recombination enzyme, a regulatory protein and a putative hemolysin activator protein with identities of 97, 95, 100 and 100%, respectively. The two remaining fragments were a hypothetical protein and an unknown protein with identities of 97 and 99%, respectively.

The results of the two sets of multiplex PCRs are shown in figure 3. All the six fragments were present in 12 of the 45 strains and a minimum of three fragments present in

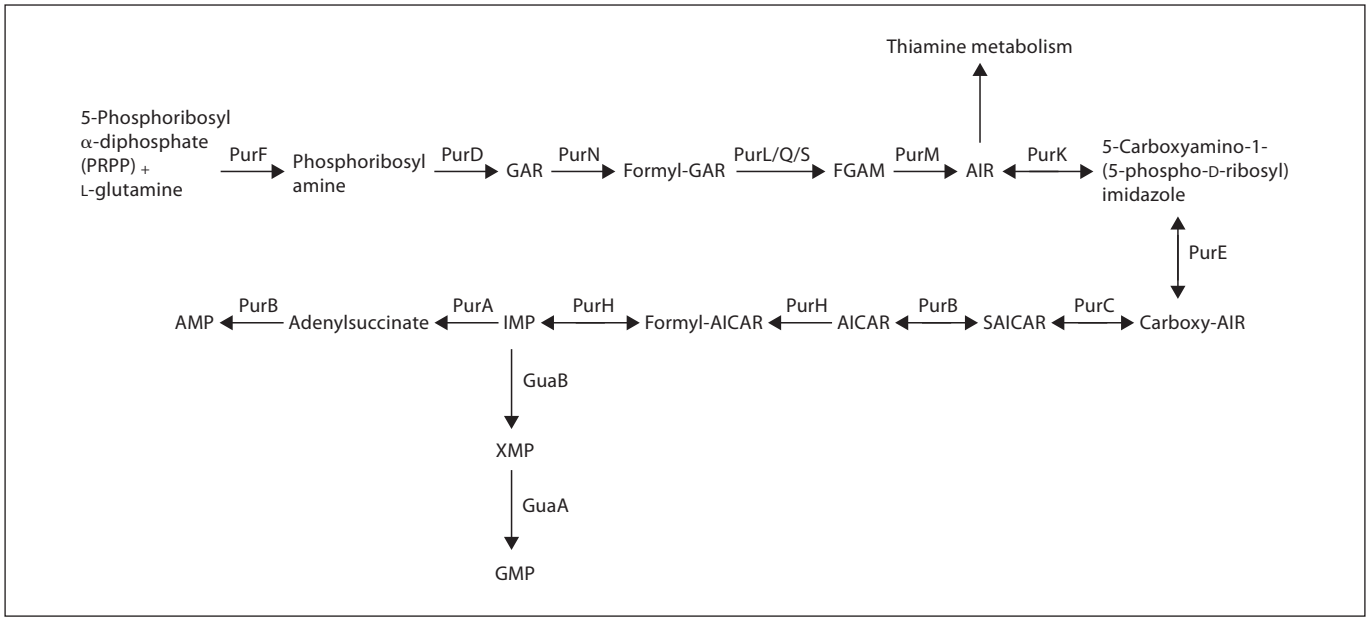

Fig. 4. Purine biosynthetic pathway in *B. pseudomallei (v)* determined by in silico analysis [Jenkins et al., 2011].

Table 4. Distribution of 'virulence determinants' in 45 clinical isolates of *B. pseudomallei*

Virulence determinant	*B. pseudomalle*i		
	blood culture (n = 24)	non-blood culture (n = 21)	total (n = 45)
Transcriptional regulator (F1)	20/24	17/21	37/45
Hypothetical protein (F2)	16/24	14/21	30/45
Unknown protein (F3)	22/24	19/21	41/45
Plasmid recombinant enzyme (F4)	15/24	11/21	26/45
Regulatory protein (F5)	18/24	17/21	35/45
Putative hemolysin activation protein (F6)	21/24	17/21	38/45

9 of the 45 strains indicating that a combination of at least three of the virulence determinants identified were present in all of the 45 clinical isolates of *B. pseudomallei*. The distribution of the six fragments in the 45 clinical isolates (possible virulence determinants) is shown in table 4.

We have demonstrated by the SH technique, that six DNA fragments present in the original virulent strain of *B. pseudomallei (v)* were significantly absent in the attenuated strain *(av)*. The six DNA fragments were recognized as part of genes encoding proteins with homology to XRE family of transcriptional regulator, a hypothetical protein, an unknown protein, plasmid recombination enzyme, regulatory protein and a putative hemolysin activation protein. A previous SH study between *B. pseudomallei* and a closely related non-infectious environmental organism *B. thailandensis* revealed capsular polysaccharide as a virulence determinant in *B. pseudomallei* [Reckseidler et al., 2001] but our study did not demonstrate this.

XRE acts as repressor of defective prophage of *Bacillus subtilis* 168 (PBSX) and is necessary for the maintenance of the bacterial lysogenic state [Wood et al., 1990]. The hypothetical protein was predicted to be a member of the nitroreductase family protein that is involved in xenobiotic biodegradation and metabolism as shown by the PATRIC database. Previous studies reported that nitro-

reductase proteins can be found in bacterial species such as *Helicobacter pylori, Klebsiella* sp. and *Salmonella typhimurium* with their important roles in mediating nitrosubstituted compound toxicity and antibiotic resistance [Jenks and Edwards, 2002; Kim and Song, 2005; Yanto et al., 2010]. Accordingly, we hypothesize that the predicted nitroreductase family protein in *B. pseudomallei* may be involved in bacterial adaptation processes.

B. pseudomallei (v) carried a gene encoding for a plasmid recombination enzyme which plays a role in plasmid maintenance, and also contributes to the distribution of small antibiotic resistance plasmids among bacteria [Wood et al., 1990]. Another putative virulent determinant is a regulatory protein and it is predicted to play a similar role as a phosphoribosylaminoimidazole carboxylase, catalytic subunit *purE* involved in purine metabolism (fig. 4). Interestingly, previous studies have reported that a purine metabolism pathway is required for virulence in a variety of pathogens such as *Staphylococcus aureus, Vibrio vulnificus* and *Bacillus anthracis* [Jenkins et al., 2011; Kim et al., 2003; Mei et al., 1997], and Crawford et al. [1996] demonstrated that mutant strains defective in *purE* gene from *Brucella melitensis* had reduced virulence in murine models. Hence, further studies of this regulatory protein may provide insights into the pathogenesis of *B. pseudomallei* infections.

The function of the unknown protein is not predictable from the current database but the hemolysin activation protein might play an important role in the regulation of hemolysin, which appears to represent a virulence factor in some human pathogens such as *Mycobacterium avium* [Maslow et al., 1999] and *Listeria monocytogenes* [Cotter et al., 2008]. Overall, all the six proteins may possibly play significant roles in the virulence and pathogenesis of melioidosis since they were absent in the attenuated strain *(av)* which we believe had reduced virulence after several subcultures and long-term storage in the laboratory.

Multiplex PCR carried out on 45 clinical isolates interestingly showed that a combination of at least three of the six virulence determinants identified were present in all these isolates. Fragment 3 was the most common being present in 41 of the 45 (91%) isolates encoding for the unknown protein according to BlastX results. Fragment 4, a plasmid recombinant enzyme, was the least common and was present only on 26 of the 45 (58%) isolates. These possible virulence determinants were fairly equally distributed between both the blood and non-blood isolates except for F4 and F6 which had differences of four fragments between the two types of isolate.

In conclusion, genomic SH method proved to be useful, whereby we have demonstrated six strain-specific DNA fragments of virulent *B. pseudomallei (v)* and absence of these in the attenuated *(av)* strain. Although a concrete functional correlation between these six putative virulence determinants and *B. pseudomallei* has yet to be confirmed, our preliminary in silico analysis results suggest that all appear to be of importance, being present in the clinical isolates. Further studies are required to fully characterize and identify their role(s) in the pathogenesis of melioidosis.

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