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PCR-BASED IDENTIFICATION OF *Burkholderia pseudomallei*

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SUMMARY

DNA amplification techniques are being used increasingly in clinical laboratories to confirm the identity of medically important bacteria. A PCR-based identification method has been in use in our centre for 10 years for *Burkholderia pseudomallei* and was used to confirm the identity of bacteria isolated from cases of melioidosis in Ceará since 2003. This particular method has been used as a reference standard for less discriminatory methods. In this study we evaluated three PCR-based methods of *B. pseudomallei* identification and used DNA sequencing to resolve discrepancies between PCR-based results and phenotypic identification methods. The established semi-nested PCR protocol for *B. pseudomallei* 16-23s spacer region produced a consistent negative result for one of our 100 test isolates (BCC #99), but correctly identified all 71 other *B. pseudomallei* isolates tested. Anomalous sequence variation was detected at the inner, reverse primer binding site for this method. PCR methods were developed for detection of two other *B. pseudomallei* bacterial metabolic genes. The conventional *lpxO* PCR protocol had a sensitivity of 0.89 and a specificity of 1.00, while a real-time *lpxO* protocol performed even better with sensitivity and specificity of 1.00, and 1.00. This method identified all *B. pseudomallei* isolates including the PCR-negative discrepant isolate. The *phaC* PCR protocol detected the gene in all *B. pseudomallei* and all but three *B. cepacia* isolates, making this method unsuitable for PCR-based identification of *B. pseudomallei.* This experience with PCR-based *B. pseudomallei* identification methods indicates that single PCR targets should be used with caution for identification of these bacteria, and need to be interpreted alongside phenotypic and alternative molecular methods such as gene sequencing.

KEYWORDS: *Burkholderia pseudomallei;* Laboratory identification; PCR; Melioidosis.

INTRODUCTION

The facultative intracellular bacterial pathogen *Burkholderia pseudomallei* causes a potentially fatal infection known as melioidosis which has become established as an endemic disease in north-eastern Brazil[14]. The most reliable means of establishing an aetiological diagnosis is isolation of *B. pseudomallei* from clinical samples[9]. Though this species is easily grown under standard laboratory conditions, it can be difficult to achieve a definitive identification. Diagnostic laboratories that regularly encounter *B. pseudomallei* have found phenotypic identification methods such as substrate utilisation panels unreliable for confirmation species identity[2,4]. Centres such as our own have come to rely on genotypic methods for identification of *B. pseudomallei*. The first such test used for this application was a semi-nested PCR protocol developed almost a decade ago[8]. This protocol enabled assembly of a collection of *B. pseudomallei* and other *Burkholderia* species, benchmarked against type culture collection strains and imported *B. pseudomallei* strains from other research centres. Our Burkholderia Culture Collection has been genotyped by EcoR1 ribotyping and Xba1 DNA macrorestriction analysis[5]. The increasing genetic complexity of the genus *Burkholderia* made us doubt the wisdom of relying on a single PCR product to confirm the identity of new clinical isolates of *B. pseudomallei*. In a previous study we therefore evaluated the relative performance of various phenotypic identification methods, used in conjunction with the established PCR protocol[7]. In the present study we sought to compare conventional and real time PCR identification protocols with the original PCR method so that we could resolve discrepancies between genotypic and phenotypic identification methods.

MATERIALS AND METHODS

Bacterial strains: The *Burkholderia* strains used in this study were described previously[7]. Briefly they include 71 *B. pseudomallei*, 19 *B. cepacia*, three *B. thailandensis*, and one each of *B. multivorans* and *B. vietnamiensis*. In addition, a *B. pseudomallei* isolate designated NCTC 10276 originating from India was included for *phaC* sequencing.

Resuscitation: Bacteria were resuscitated by subculture onto 5% horse blood agar and incubated for 24 h at 37 °C to give single colony growth. A single blood agar plate was used for each isolate, and stock cultures were spread to produce single-colony growth in the third or

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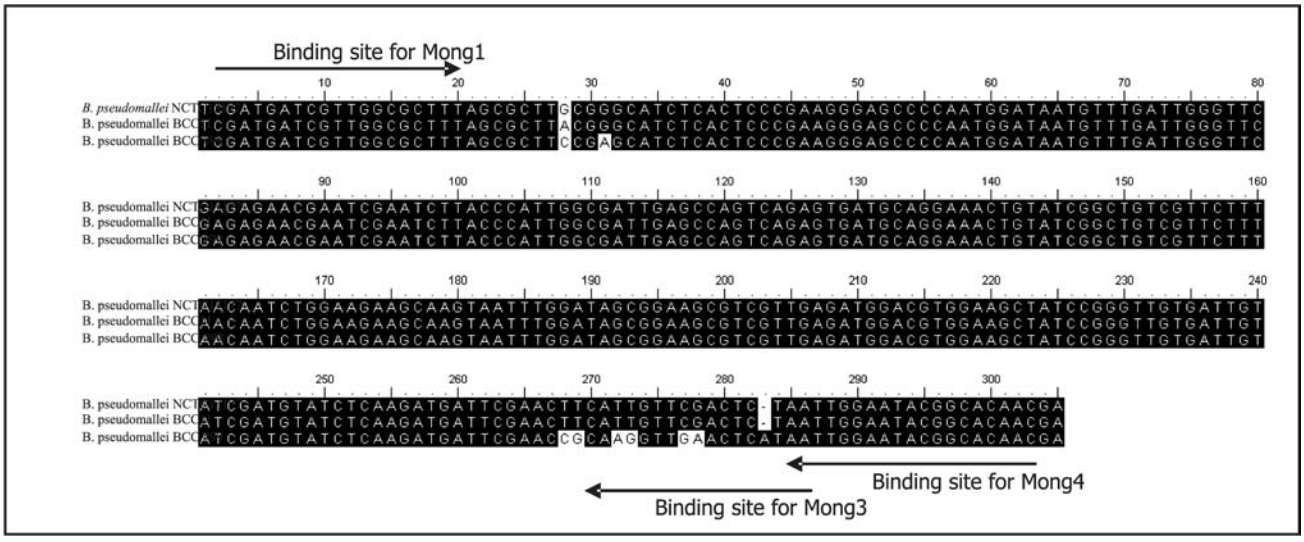

Fig. 1 - Clustal alignment of sequenced 16s-23s spacer PCR first round product for *B. pseudomallei* NCTC 13177, BCC11 and BCC #99. Arrows indicate annealing sites for the primers used in the 16s-23s spacer identification assay.

fourth quadrant. A single colony was then sub-cultured onto 5% horse blood agar and incubated at 37 °C for 24 h to produce pure growth for all subsequent tests.

DNA preparation: A single colony of *B. pseudomallei* grown on blood agar (Excel Laboratories, Australia) was re-suspended in diethylpyrocarbonate treated deionized water. The suspension was heated at 100 °C for 15 min and centrifuged at 9000 x g to pellet the cell debris. The supernatant was used as the template for all subsequent PCR assays.

PCR targets, mixes and cycling conditions: All primer design was conducted using Primer Express software (Applied Biosystems). Table 1 shows the primer pairs used in this study, their binding site relative to the start codon of the gene of interest and the annealing temperature for their respective PCR programs. The Taqman probe used for realtime detection of *lpxO* was synthesized by Biosearch Technologies inc. (Novato, Ca 94949, USA) and consisted of a 5' 6-FAM reporter label and a 3' 'Black Hole Quencher'. Semi-nested PCR was performed as described previously[7]. *phaC* PCR was performed using the following PCR mix conditions; 1 unit(u) Amplitaq Gold Enzyme (Applied Biosystems), PE PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM each dNTP and 0.5 µM of each primer in a total volume of 20 µL (including 8 µL of template). *lpxO* PCR was performed using the following PCR mix conditions; 0.5 u Amplitaq Gold Enzyme (Applied Biosystems), PE PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM each dNTP and 0.4 µM of each primer in a total volume of 20 µL (including 8 µL of template). Realtime *lpxO* PCR was performed using the following PCR mix conditions; 0.75 u Amplitaq Gold Enzyme (Applied Biosystems), PE PCR buffer, 4.0 mM $MgCl_2$, 0.2 mM each dNTP and 0.2 µM of each primer and 0.1 µM of Taqman probe in a total volume of 20 µL (including 8 µL of template). All other conventional PCR methods were performed under the following conditions, pre-PCR of 10 minutes at 95 °C to fully denature the template DNA and activate the polymerase followed by 45 cycles of 30 sec at 94 °C for denaturation, 30 sec at the

Table 1
Primers and probes used for all PCR

Target	Name	Primer (5'-3')	Binding site (aa)	Annealing temperature (°C)
16s-23s spacer	Bp1	CGATGATCGTTGGCGCTT	397*	50
1st round	Bp4	CGTTGTGCCGTATTCCAAT	680*	
16s-23s spacer	Bp1	CGATGATCGTTGGCGCTT	397*	55
2nd round	Bp3	ATTAGAGTCGAACAAT	666*	
lpxO PCR	Sabp76F	GCGCCGMTCAATWKKTTCG	112	55
	Sabp554R	GGCCCARTGCAGSTARGTCTCGT	554	
lpxO RealTime	Burk-1F	TTGTTTCGCCTATGCGTTCTC	123	60
	Burk-2R	CCACTCGCGCTTGAGGAT	196	
Taqman® probe	Burk-110	ACGTGCCGAACACGCCGTATATCG	146	
phaC	Gene1-1108	TGCGGCAGGGGATGAGAA	1108†	55
	Gene1-1510	GCAGAAGCCCAGCGTGTTGA	1510†	

* Binding site is numbered using 16s start codon as reference; † binding site is relative to *phaC* start codon of DSMZ9242 (accession number AF153086)

appropriate annealing temperature and 45 sec at 72 ºC for extension. Following the final cycle the samples were maintained at 72 ºC for a further seven min. PCR products were demonstrated by Ethidium bromide gel electrophoresis on 2.5% agar gels. Digital gel images were captured and optimized for brightness and intensity using UVIdoc capture system (Cambridge, UK). The *lpxO* realtime PCR protocol was performed under the following conditions, pre-PCR of 10 minutes at 95 ºC to fully denature the template DNA and activate the polymerase followed by 50 cycles of 10 sec at 94 ºC for denaturation, 10 sec at the appropriate annealing temperature and 30 sec at 72 ºC for extension. Amplification and detection was carried out in an Applied Biosystems Prism 7000 Sequence Detection System.

Sequencing and analysis: Sequencing was carried out on the products from the first round of semi-nested PCR, *lpxO* PCR and *phaC* PCR in the forward and reverse direction. The PCR products were treated with pre-sequencing clean-up enzyme (ExoSap-It USB Corp., Cleveland, Ohio) and then used as the template in a sequencing mix (Applied Biosystems, BigDye terminator v3.1). The now labeled products of the sequencing reaction were then filter purified using Microcon PCR filters (Amicon Millipore, North Ryde, Australia) and sequenced in a 3100 *Avant* genetic analyzer (Applied Biosystems). Sequences were checked for the presence of ambiguities and discrepancies between the forward and reverse read using Chromas v2.3 (Technelysium Pty Ltd, QLD, Australia). Where ambiguities or discrepancies could not be resolved, the PCR product was re-sequenced until a clear read was obtained. The sequences were then aligned using ClustalX v1.83[15] and presented in graphic format using Bioedit v7.0.0 (Isis Pharmaceuticals Inc.)[3]. Lastly, phylogenetic analysis of *phaC* gene alignments was conducted using Phylodraw v0.82 (Pusan National University, South Korea).

RESULTS

16S-23s spacer PCR: In our previous study we reported the observation that one isolate, (BCC#99) displayed discrepancies between its phenotype and the results of the semi-nested PCR used as a reference. In the present study, closer analysis of the semi-nested PCR determined that no amplification occurred in the second round of amplification, despite generation of PCR product in the first round. The results of sequencing the first round product are displayed as a sequence alignment and shown in Fig. 2. We noted that BCC#99 contained four nucleotide substitutions and one nucleotide insertion at the binding site for the reverse primer of the second round PCR (Mong3). With this one exception, the established 16s-23s spacer PCR method correctly identified the isolates used in the present study (Table 2).

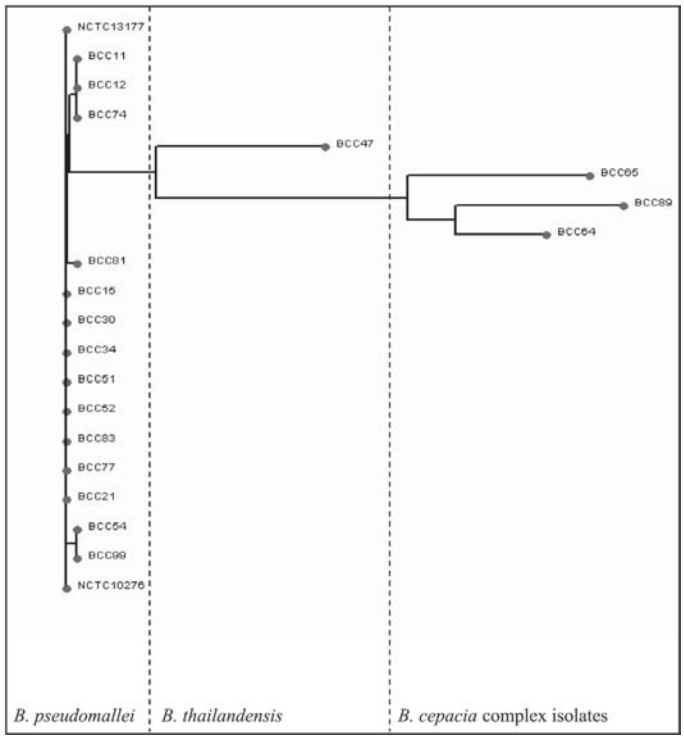

Fig. 2 - Phylogram of 20 representative *Burkholderia* spp. including 16 *B. pseudomallei* based on DNA sequence of PCR-amplified *phaC* gene fragments. Other isolates: BCC47 = *B. thailandensis*, BCC64 = *B. cepacia*, BCC65 = *B. multivorans* and BCC89 = *B. vietnamensis*.

***lpxO* conventional PCR & sequencing *lpxO* realtime PCR:** When we used conventional PCR primers targeting a 900bp *B. pseudomallei* homologue of the *S. typhimurium lpxO* gene to probe our culture collection, we detected the corresponding PCR product in 64 of 72 *B. pseudomallei* isolates and in none of the non-pseudomallei *Burkholderia* isolates (Table 2). Sequencing of the *lpxO* PCR products allowed adaptation and optimization of the method to real-time PCR which subsequently identified 72 of 72 *B. pseudomallei* isolates tested and resulted in no false positives. The previously PCR-negative discrepant isolate BCC99 was positive by this method. This assay correctly identified all *B. pseudomallei* isolates and excluded near-neighbour species in approximately 1/3 the time taken by the original semi-nested method. The semi-nested assay took approximately six hours to complete, including one hour of technician time. The realtime assay produced results in two hours with only 30 min of technician time.

Table 2
Results of all PCR assays by species

Species (n)	No. (%) of positive results					Total
	B. pseudomallei (72)	*B. cepacia* (18)	*B. vietnamiensis* (1)	*B. thailandensis* (3)	*B. multivorans* (1)	(95)
16s-23s Spacer	71	0	0	0	0	71
lpxO PCR	64	0	0	0	0	64
*lpxO*Taqman®	72	0	0	0	0	72
phaC PCR	72	15	1	3	1	91

(n) denotes total number of isolates tested for each species.

Table 3
Table of percentage identity between studied *Burkholderia* species of the *phaC* gene fragment

	B. vietnamiensis	*B. thailandensis*	*B. multivorans*	*B. cepacia*
*B. pseudomallei**	87.9	94.5	88.8	89.6
B. cepacia	94.5	87.7	93.1	
B. multivorans	91.2	97.2		
B. thailandensis	86.6			

*The majority sequence type was used as the reference sequence for comparison with *Burkholderia* spp. other than *B. pseudomallei*.

***phaC* PCR and sequencing:** The PCR method designed to amplify a fragment of the *phaC* gene detected product in all but three of the isolates tested, none of which were *B. pseudomallei* (Table 2). The *phaC* PCR product was sequenced in 20 distinct strains; 16 *B. pseudomallei* and one each of *B. thailandensis*, *B. vietnamiensis*, *B. cepacia* and *B. multivorans*. Fig. 2 shows a phylogenetic tree using the sequences generated. Table 3 shows the percentage identity between *pha*C PCR product sequence in the *B. pseudomallei* isolates and the other *Burkholderia* species. Of the 16 *B. pseudomallei* isolates that had *phaC* PCR product sequenced, 10 were indistinguishable and the remaining six isolates differed by not more than 0.5% from this majority sequence type. The most closely positioned non-pseudomallei *Burkholderia* sp. was *B. thailandensis* with 94.3% identity to the majority *B. pseudomallei* sequence type, followed by *B. cepacia*, *B. multivorans* and *B. vietnamiensis*.

DISCUSSION

Previous evaluations of *B. pseudomallei* identification methods have relied on assumptions about the sensitivity and specificity of the reference method or the culture collection used to validate the identification approach taken[4,10]. In our recent evaluation of confirmatory phenotypic methods, we regarded the semi-nested PCR protocol used during assembly of the *B. pseudomallei* culture collection as 100% reliable. The isolate known as BCC #99 was therefore classified according to the results of a *B. cepacia* PCR-based identification method, despite the weight of confirmatory phenotypic evidence, which favored *B. pseudomallei*[7]. Discrepancies of this kind need careful investigation in order to exploit opportunities to improve the effectiveness of genotypic identification tests. Closer examination of the PCR-based identification test products indicated that while the PCR was occurring in the first round of amplification, none occurred in the second round. When we sequenced the first round PCR product, we found that nucleotide deletions at the second round reverse primer binding site produced an aberrant, under-sized first round product and were sufficient to prevent the primer from annealing. This caused the false negative PCR result when evaluating the first or second round products. In view of persuasive confirmatory phenotypic data and the degree of homology between the remainder of the sequenced PCR product and an NCTC strain, we believe the definitive identification of BCC #99 should be changed from *B. cepacia* to *B. pseudomallei*.

This newly observed deficiency in the semi-nested method we have used for six years suggested the need for an alternative PCR-based *B. pseudomallei* confirmatory method. As none of the published methods were ideally suited to the role we previously identified for PCR in our clinical laboratory *B. pseudomallei* discovery pathway, we developed two candidate protocols *de novo*. Drawing on phenotypic analyses in which the fatty-acid 2-hydroxymyristate was almost exclusively produced by *B. pseudomallei*[6], we identified a potential homologue to a gene used by *S. typhimurium* in the synthesis of 2HMA[1]. In the present study, a real time PCR protocol developed to detect the *B. pseudomallei lpx*O homologue correctly distinguished between all tested *B. pseudomallei* and non-pseudomallei *Burkholderia* sp. isolates without error. The performance of this real time PCR protocol indicates that it may be a candidate for incorporation in multiplex PCR protocols for diagnostic and biosecurity applications, subject to more extensive testing against a larger number of geographically diverse *B. pseudomallei* isolates and a wider range of bacteria from other genera.

Sequence variation around the primer binding sites for the *B. pseudomallei lpxO* product was minimal, and further adds to the suitability of the method as a confirmation of identity. But it also indicates that sequencing of the *lpxO* PCR product is unlikely to assist genetic subtyping. However, the product of the *phaC* PCR method we developed was more suitable for molecular subtyping for the same reason.

The polyhydroxybutyrate accumulation pathway is highly conserved in the Burkholderias. The *phaC* gene encodes the polyhydroxybutyrate synthase; the enzyme that polymerizes β-hydroxybutyrate monomers into PHB[12]. Using the *phaC* gene sequence of *Burkholderia* species DSMZ9242 (AF153086) as a starting point[13], preliminary alignments of *phaC* genes from *Burkholderia* species and other non-*Burkholderias* indicated the presence of both conserved and non-conserved regions, suggesting that it might be a suitable identification target for attribution of genus and species status. From our study it is clear that the sequence we chose to amplify is conserved across a wider range of *Burkholderia* spp. than *B. pseudomallei*. The PCR protocol was therefore unsuited as an aid to species level identification. However, nucleotide sequence analysis of the *phaC* PCR product gave a high level of phylogenetic discrimination between different *Burkholderia* species.

The role for PCR-based methods in a *B. pseudomallei* laboratory discovery pathway is evident. The real time *lpxO* PCR protocol is fast enough to be used for same-day confirmation of presumptive *B. pseudomallei* in suitably equipped diagnostic laboratories. Where only conventional PCR is available, the conventional PCR method is still fast enough to replace the confirmatory role proposed for gas liquid chromatography of bacterial fatty acid methyl ester derivatives[7]. In the absence of other confirmatory methods; whether phenotypic or

genotypic, two complementary PCR-based protocols should be employed. Despite promising early results from 16S sequencing as a *B. pseudomallei* identification method, sequence from the 16s locus does not always discriminate between *B. pseudomallei* and other *Burkholderia* species. Sequencing of *lpxO* and other Burkholderia-specific gene loci may be necessary to resolve equivocal, ambiguous or otherwise confusing laboratory identification test results. An increased reliance on molecular methods to confirm the identity of *B. pseudomallei* will set the scene for better quality assurance of presumptive identification tests used in a field setting. Several other real time PCR protocols have been described for identification of *B. pseudomallei* using targets in the 16s rDNA, *fliC*, and Type Three Secretion genes[15,17,18,19,20]. While a study comparing the performance of these assays has yet to be performed it is likely that one or more will find a place in an expanded laboratory discovery pathway, possibly being incorporated into a multiplex real time PCR method.

This investigation of discrepancies between *B. pseudomallei* PCR-based and phenotypic identification demonstrated a failure of primer annealing at the reverse primer binding sites in the original PCR protocol. Due to the observed failure and relatively long time to result of the 16s-23s nested method its use was discontinued and a suitable alternative real-time PCR-based identification protocol targeting a gene locus specific to *B. pseudomallei* and associated with bacterial acid fatty acid metabolism was developed. PCR methods currently incorporated into *B. pseudomallei* discovery pathways for use in clinical diagnostic laboratories should be interpreted alongside other identification tests, and not relied upon as a stand-alone identification method.

RESUMO

Identificação de *Burkholderia pseudomallei* baseada em PCR

As técnicas de amplificação de DNA estão sendo cada vez mais utilizadas em laboratórios clínicos para a confirmação da identificação de bactérias que têm importância médica. Um método de identificação de *Burkholderia pseudomallei* baseado em PCR tem sido usado em nosso centro há 10 anos e foi utilizado para confirmar a identificação de bactérias isoladas de casos de melioidose no Ceará desde 2003. Este método particular tem sido usado como padrão ouro para métodos menos discriminatórios. Nesse estudo, avaliamos três métodos de identificação de *B. pseudomallei* baseados em PCR e usamos seqüenciamento de DNA para solucionar discrepâncias entre os resultados baseados em PCR e os métodos de identificação fenotípica. O estabelecido protocolo de PCR semi-nested para a região espacial 16-23s da *B. pseudomallei* produziu um consistente resultado negativo para um de nossos 100 isolados testados (BCC#99), mas identificou corretamente todos os outros 71 isolados de *B. pseudomallei*. Uma variação anômala da seqüência foi detectada na região interna do sítio de ligação do primer reverso para este método. Métodos de PCR foram desenvolvidos para a detecção de outros dois genes bacterianos metabólicos de *B. pseudomallei*. O protocolo de PCR *IpxO* convencional teve sensibilidade de 0,89 e especificidade de 1,0, enquanto que o PCR em tempo real mostrou-se ainda melhor, com sensibilidade de 1,0 e especificidade de 1,0. Este método identificou todos os isolados de *B. pseudomallei*, incluindo o isolado discrepante que teve o PCR negativo. O protocolo de PCR phaC detectou o gene de todos os *B. pseudomallei* e em todos exceto três isolados de *B. cepacia*, tornando este método de identificação de *B. pseudomallei* baseado em PCR inadequado. Esta experiência com métodos de identificação de *B. pseudomallei* baseados em PCR indica que devemos ter precaução quando estes forem utilizados sozinhos para identificação dessa bactéria e que eles necessitam ser interpretados em conjunto com métodos fenotípicos e moleculares alternativos, tais como seqüenciamento genético.

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