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Development and Validation of Conventional PCR for the Detection of the sctQ Gene

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Abstract

Burkholderia pseudomallei can potentially cause lethal infections if not diagnosed properly. At present, several PCR assays have been developed for *B. pseudomallei* identification, with variable sensitivities and specificities. In this report, a PCR assay targeting a broad-range *B. pseudomallei* highly conserved gene of the type three secretion system-1 (TTS1) cluster was developed and was validated against a panel of multi-locus sequence types of clinical *B. pseudomallei* isolates. The analytical sensitivity and specificity were 100 % each. The lower limits of detection of purified DNA and bacterial cells spiked in blood specimens were 100 fg/µL and 18.4 x 10^5 CFU/mL, respectively. This study demonstrates the utility of the TTS1 gene for the *B. pseudomallei* identification.

Keywords: Melioidosis, *Burkholderia pseudomallei*, PCR, TTS1, MLST, sctQ gene

1. Introduction

Melioidosis is a disease of varying severity that affects both humans and animals, and that can be fatal if left untreated (Podnecky NL, *et al.*, 2013). It is caused by *Burkholderia pseudomallei* that can be readily recovered from the water and soil in endemic areas, such as Northern Australia and Southeast Asia (Puthucheary SD, 2009). The clinical presentation of melioidosis includes non-septicemic subclinical infection, cutaneous lesions, or severe septicemia that may disseminate to a single or multiple organs (Zueter *et al*., 2016a). In an acute infection state, death may occur within twenty-four to forty-eight hours of the onset of symptoms. The mortality rate can reach up to 40 %. In addition, *B. pseudomallei* is intrinsically resistant to many of the broad spectrum antibiotics (Haase *et al.*, 1998, Podnecky *et al.*, 2013). Delay in the isolation and identification of *B. pseudomallei* contributes to the high mortality rates in more than 50 % of the patients during the first two days after hospital admission and before obtaining positive bacterial culture results (Dharakul *et al.*, 1996).

At present, several PCR assays have been developed for *B. pseudomallei* identification, with variable sensitivities and specificities. It was suggested that a gene targeted by a PCR assay might not be present among all *B. pseudomallei* isolates, which may compromise detection performance (Novak *et al.*, 2006). The type three secretion system-1 (TTS1) gene cluster was reported as being universally present among *B. pseudomallei* (Winstanley and Hart, 2000, Novak *et al.*, 2006). In this study, a PCR assay targeting the highly conserved TTS1 cluster was developed and validated against a panel of MLST-strain types of *B. pseudomallei*.

2. Materials and Methods

2.1. Primers Design

In this study, a previously identified area located adjacent to the *orf1-stcQ* gap in the TTS1 gene cluster (GenBank accession code: AF074878) (Holden *et al.*, 2004) was targeted for PCR amplification based not only on its *B. pseudomallei* theoretical specificity, but also on its apparent ubiquitous distribution among clinical and environmental isolates (Smith-Vaughan *et al.*, 2003). Forward and reverse primers were constructed using Primer-Blast service www.ncbi.nlm.nih. gov/tools/primer-blast/. Forward and reverse primers target a 316-bp region located in putative gene *sctQ* (29 to 344) and on TTS1 (24015 to 24433): *sctQ*F (5`-CACACTTCAACG CGACACTG-3`) and *sctQ*R (5`-GGGAGCTCGATG ACATAGCC-3`). Primers were synthesized and provided as desalted lyophilized form from 1stBASE, Singapore.

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2.2. Collection and Preparation of Bacterial Strains

The study utilized thirty-eight archived and newly-recovered *B. pseudomallei* isolates from different clinical samples obtained from melioidosis patients admitted to Hospital Universiti Sains Malaysia, at Kelantan state from 2007 to 2014. Bacteria were reactivated by aerobic cultivation on Tryptone soya agar (Oxoid Ltd., Basingstoke, United Kingdom). Similar reactivation procedure was applied on archived non-*B. pseudomallei* bacteria that were obtained from the stock culture unit of Medical Microbiology and Parasitology Department at Universiti Sains Malaysia. The study was approved by the Research Ethics Committee (Human) (USM/JEPeM/ 15110495).

2.3. DNA Extraction

Dense bacterial suspensions were prepared in 10 mL Tryptone soya broth and were subjected to genomic DNA extraction using DNeasy tissue kit (Qiagen Inc., Hilden, Germany) according to the manufacturer instructions. In addition, DNA from other bacteria, fungi, and from human blood was prepared. For detection limit determination, 10-fold serial dilution of extracted *B. pseudomallei* DNA was made in elution buffer beginning from 1 ng/µL to 1 fg/µL. For spiked samples, 2 mL EDTA-blood was centrifuged at 1500 g for ten minutes, and 200 µL of the resultant buffy coat were subjected to DNA extraction using the QIAamp DNA blood mini kit (Qiagen Inc., Hilden, Germany) according to the manufacturer instructions. In both protocols, the final incubation in the elution step was extended to thirty minutes to increase the DNA yield.

2.4. PCR Assay

The optimized PCR mixture contained 0.5 µL of extracted DNA in 25 µL final reaction volume consisting of PCR mastermix containing 1x MyTaq Red reaction buffer (Bioline Ltd UK), comprised of 5 mM dNTPs, 15 mM $MgCl_2$, stabilizers and enhancers, and 1 U/µl MyTaq DNA polymerase (Bioline Ltd UK), a final concentration of 0.2 µM of each forward and reverse primers, and PCR water. Amplification was performed with Master Cycler Nexus Gradient (Eppendorf, Germany) using standard settings. The optimized thermal profile was initial denaturation at 95ºC for four minutes, followed by thirty cycles of 95ºC:15 s, 65ºC:15 s, and 72ºC:30 s, and a final extension at 72ºC:4 min. No-template (PCR water) and positive *B. pseudomallei* controls were included in each run to rule out amplification failure or possible contamination.

An internal control pET32a plasmid and its primers (T7 Promoter780-764 5' TAATACGACTCACTATAG 3' and T7 Terminator 168-185 5' GCTAGTTATTGCTCAGCGG 3'), provided by Novagen, USA, were added to rule out the possibility of amplification inhibition induced by clinical specimen constituents. Plasmid volume and the final concentrations of its primers were suited to run in duplex with the *sctQ*-PCR. Using 1.5 % agarose gel loaded with Gelred stain (Bioline Ltd UK), PCR products along with DNA ladder (10kb Hyperladder™I, Bioline Ltd, UK) electrophoresis was performed and the separated amplicons were visualized by ultraviolet light transilluminator and computerized image analysis system (G-Box, Syngene, USA).

2.5. PCR Assay Sensitivity

For sensitivity and specificity, the concentration of all tested DNA templates was normalized at 1 ng/µL using NanoDrop UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE). The genetic and geographic spectrum of detection of the sctQ-PCR assay was assessed using DNA from 83 *B. pseudomallei* isolates encompassing thirty-two different genotypes obtained from different distinct population communities of peninsular Malaysia. In addition, up to half of the isolates' genotypes were previously identified in other surrounding south-eastern countries, and most of them have suggested Australian ancestry. The isolates were provided by Zueter *et al* (2018).

2.6. PCR Specificity

Discriminative specificity of the *sctQ*-PCR assay was determined by screening against closely related species including *Burkholderia* spp. and *Pseudomonas* spp. We also evaluated the assay using clinically important non-*Burkholderia* bacteria and eukaryotic species (Table 1).

Table 1. Organisms* (*n=72*) used to test the specificity of the *sctQ*-PCR assay

Organism category	Name of organism	Count tested
Genetic relatives (n=27)	*Burkholderia cepacia KR869104*	2
	Burkholderia cepacia	9
	Burkholderia thailandensis KR869105 (ST77)	1
	Pseudomonas aeruginosa	7
	Pseudomonas lutelo,	1
	Pseudomonas stutzeri	1
	Pseudomonas flurosence	1
	Stenotrophomonas maltophilia	1
	Acinetobacter baumannii	1
	Chryseobacterium indologens	1
	Chromobacterium violaceum	1
	Moraxella catarrhalis	1
Other types of bacteria (n= 37)	*Shigella boydi,*	1
	Shigella dysentri,	1
	Shigella sonnie	1
	Shigella flexeri	1
	Salmonella enterica	1
	Salmonella typhi	1
	Salmonella paratyphi	1
	Proteus merabillis	1
	Proteus volgaris	1
	Escherichia coli	1
	Citrobacter freundi	1
	Klebsiella pneumoniae	1
	Enterobacter cloacae	1
	Staphylococcus aureus	2
	Streptococcus pneumoniae	2
	Streptococcus group A	1
	Streptococcus group B	1
	Streptococcus group G	1
	Streptococcus group D	1
	Listeria monocytogens	1

	Mycobacterium tuberculosis	3
	Mycobacterium bovis	1
	Haemophilus influenza	2
	Neisseria meningitides	1
	Liptospira interrogans	1
	Aeromonas hydrophila	1
	Helicobacter pylori	4
	Vibrio mimicus	1
	Vibrio cincinnatiensis	1
Other organisms (n=8)	Human blood cells	4
	Candida albicans	1
	Candida tropicalis	1
	Candida glabrata	1
	Cryptococcus neoformans	1

*Identification of isolates was confirmed by biochemical tests. MLST and 16S rRNA typing were done for *Burkholderia species*.

2.7. The Lower Limit of Detection

Ten-fold serial dilution of extracted *B. pseudomallei* DNA was made in elution buffer beginning from 1 ng/µL to 1 fg/µL. Optimized *sctQ*-PCR run along with positive and negative controls was done for every dilution in duplicate. The end products were subjected to electrophoresis followed by visualization.

The PCR assay performance was further assessed by determining the lowest detectable concentration of *B. pseudomallei* cells in spiked blood samples. In a class-II biosafety cabinet, *B. pseudomallei* cells' count of 10^9 colony-forming units per milliliter of sterile phosphate buffered saline (CFU/mL), was prepared via comparison to McFarland (McF) standard. Count was confirmed by the agar-dilution method. One milliliter of the suspension was transferred into 1.5 mL microtube. The suspension was then serially diluted up to a concentration of 1.0 CFU/mL. A 100 µL aliquot from each dilution was spread onto tryptone soya agar (TSA) plates, and was incubated for seventy-two hours at 37ºC. The rest of the volume (900 µL) of each individual dilution was used to spike ten 2.0 mL EDTA anti-coagulated blood samples.

For quality control, 100 µL of PBS was plated on TSA for seventy-two hours to confirm sterility. In addition, 1 mL of sterile PBS was added to 2.0 mL EDTA blood, was mixed thoroughly, and processed along with all spiked blood samples for DNA extraction and PCR. DNA was extracted from the buffy coat. The optimized *sctQ*-PCR was applied on DNA extracted from all spiked blood samples and controls. The internal control pET32a plasmid and its primers were added to all PCR runs, to insure the absence of the inhibition of amplification reactions.

3. Results

For *in-silico* specificity, DNA sequencing for the *sctQ*-PCR purified amplicons was done and showed complete match once aligned to a reference sequence. *sctQ*-PCR showed 100 % analytical sensitivity and specificity (Figure 1). No amplification for non-*B. pseudomallei* was observed, while observing amplification bands for the internal control which confirms the absence of amplification inhibitors, and validates the negative results for non-*B. pseudomallei* controls (Figure 2).

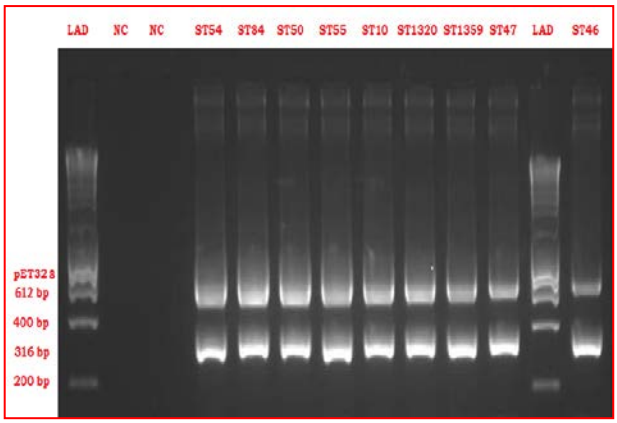

Figure 1. Gel electrophoresis of duplex PCR performed on different sequence types of *B. pseudomallei*. Gel showing amplicon bands for *sctQ* (316 bp) and for the internal control (pET328). LAD: 10kb DNA ladder; NC: negative control; ST: sequence types of *B. pseudomallei*.

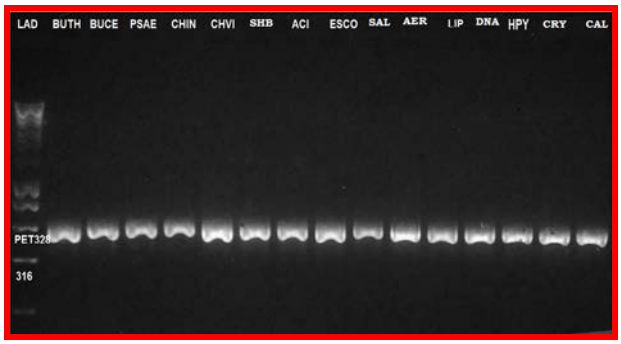

Figure 2. Duplex PCR of sctQ primers and plasmid pET328 internal control plasmid against non-B. pseudomallei isolates. LAD: 10kb DNA ladder; BUTH: Burkholderia thailandensis, BUCE: Burkholderia cepacia, PSAE: Pseudomonas aeruginosa, CHIN: Chryseobacterium indologenes, CHVI: Chromobacterium violaceum, SYB: Shigella boydii, DNA: human blood, ESCO: Escherichia coli, SAL: Salmonella enterica serovar enteritidis, AER: Aeromonas hydrophila, LIP: Leptospira interrogans, HAI: Haemophilus influenzae, HYP: Helicobacter pylori, CRY: Cryptococcus neoformans, CAL: Candida albicans.

The lower concentration of purified *B. pseudomallei* DNA that was amplified by *sctQ*-PCR was 100 fg/µL, whereas the lowest number of bacterial cells detected in spiked blood specimens was 18.2 X 10^5 CFU/mL (Figures 3 and 4).

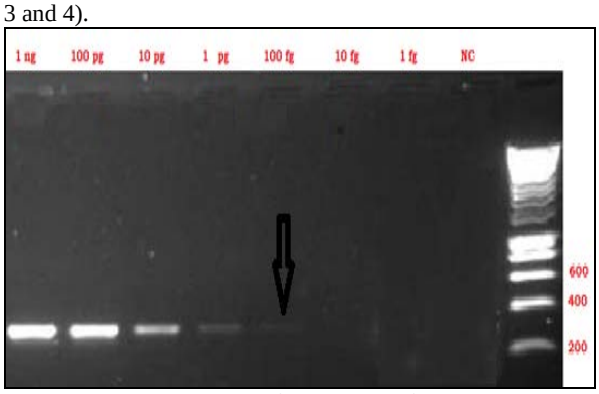

Figure 3. Gel electrophoresis of *sctQ*-PCR performed on gradient concentrations of purified *B. pseudomallei* DNA. The arrow indexes for the lower concentration of DNA detected by *sctQ*-PCR. LAD: 10kb DNA ladder; NC: negative control; The gradient concentration started from 1 ng/µL to 1 fg/µL.

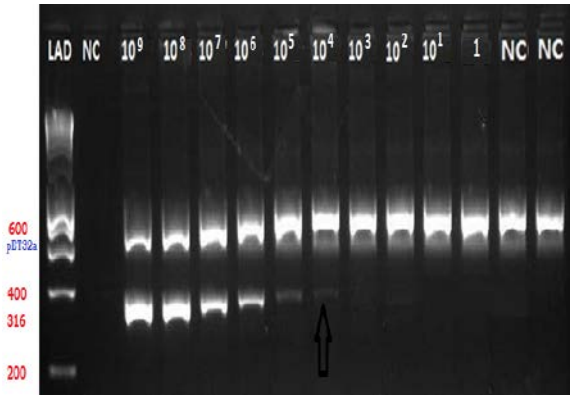

Figure 4. Gel electrophoresis of *duplex*-PCR performed on blood samples spiked with gradient inoculum of *B. pseudomallei*. The arrow indexes for the lower concentration of DNA detected by *sctQ*-PCR. LAD: 10kb DNA ladder; NC: negative control; The gradient inoculum started from 10^9 to 1 CFU/mL

4. Discussion

PCR-based diagnosis is preferred over immunoassays and shows higher sensitivity and specificity. A previously performed comparative study reported the superiority of PCR over three serological methods using samples obtained from culture-positive patients (Sermswan *et al*., 2000).

The complete sequence of the *B. pseudomallei* genome had been studied previously (Winstanley *et al*., 1999, Holden *et al*., 2004), and provided various gene targets for specific species identification using PCR assays including the type III secretion (TTS1) gene cluster (Winstanley and Hart, 2000), 16S–23S ribosomal RNA (rRNA) intergenic region (Kunakorn *et al*., 2000), rRNA spacer, 23S rRNA (Bauernfeind *et al*., 1998), 16S rRNA (Dharakul *et al*., 1996), lipopolysaccharide (LPS) gene (Rattanathongkom *et al*., 1997), flagellin C (*fliC*) and ribosomal protein subunit S21 (*rpsU*) (Tomaso *et al*., 2005).

Such genes have been utilized for the detection of *B. pseudomallei* from different clinical specimens and environmental samples, as well as pure culture, and showed good sensitivities and specificities (Brook *et al*., 1997, Rattanathongkom *et al*., 1997, Sura *et al*., 1997, Novak *et al*., 2006).

The selection of gene targets according to one strain may lead to false-negative results because a particular strain may not be representative for the global *B. pseudomallei* population (Tomaso *et al*., 2005).

On the other hand, PCR faces sensitivity and specificity issues when applied for a direct diagnosis on the patient's clinical specimens (Haase *et al*., 1998; Kunakorn *et al*., 2000; Sermswan *et al*., 2000).

Herein, the researchers reported the design, optimization, and validation of a PCR assay targeting the *sctQ* gene of the TTS1 cluster that is present among a wide range of *B. pseudomallei* strains.

For specificity testing, *B. thailandensis* and *B. cepacia* were included as relative non-*B. pseudomallei* species. *B. thailandensis* is well-known as the most genetically related to *B. pseudomallei*, and many of the pre-existing tests give false positive results (Zueter *et al*., 2016b), thus it would be of a high discriminative value for assay validation. *B. cepacia* is the most frequently isolated *Burkholderia* strain from human clinical samples along with *B. pseudomallei* (Pal 2018); therefore, it was added to the test panel to test the discriminative ability of *stcQ*-PCR.

The *sctQ*-PCR assay performance results agree with previous studies that reported excellent sensitivity and specificity of PCR when tested against purified bacterial DNA extracted from pure cultures or bacterial lysate. However, assay sensitivity decreased when testing clinical specimens due to possible effects of PCR inhibitors present in the specimens, applied specimen collection methods, or effects of antibiotics (Novak *et al*., 2006).

Many studies have evaluated different PCR assays for the detection of *B. pseudomallei* in clinical samples (Gal *et al*., 2005; Kaestli *et al*., 2012). Moreover, other comparative studies demonstrated the superiority of real-time PCR assays over conventional PCR in terms of greater analytical sensitivity, speed, and ease of use (Supaprom *et al*., 2007). Novak *et al* (2006) developed a real-time PCR assay targeting a type III secretion system for the identification of *B. pseudomallei*. They thoroughly evaluated the assay on spiked blood samples, and showed better accuracy than PCR assays previously published. Gal *et al*. (2005) have reported that the testing of a larger sample volume could improve the PCR performance for clinical samples.

A comparative study was performed for seven recently published real-time PCRs assays applied on clinical samples. The gene targets were *YLF/BTFC*, TTS1-*orf*2, TTS1-*orf*11, *IpxO*, and *mprA*, and showed that the real-time PCR assay targeting TTS1-*orf*2 was the most reliable on clinical samples with analytical and diagnostic specificity of 100 %, diagnostic sensitivity of 80 %, and a limit of detection of 5 fg/µL. In addition, the assay was useful in the detection of *B. pseudomallei* in purified samples and environmental samples (Kaestli *et al*., 2012).

5. Conclusion

PCR-based methods have significantly improved early disease diagnosis. Our developed assay showed acceptable performance compared to previous molecular assays. A larger panel of *Burkholderia species*, which was difficult to obtain in this study, is needed to confirm the discriminative ability of our assay. The end-point PCR designed in this study represents the first step on the way to the evaluation and commercialization of melioidosis molecular diagnosis in the hospital laboratory setting.

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