Comparison of TaqMan PCR Assays for Detection of the Melioidosis Agent *Burkholderia pseudomallei* in Clinical Specimens

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**Running head:** TaqMan assays to detect *Burkholderia pseudomallei*
Abstract

Melioidosis is an emerging infectious disease caused by the soil bacterium *Burkholderia pseudomallei*. In diagnostic and forensic settings, molecular detection assays need high sensitivity with low limits of detection, but also high specificity. In a direct comparison of published and newly developed TaqMan PCR assays, we found the TTS1_orf2 assay superior in detecting *B. pseudomallei* directly from clinical specimens. The YLF/BTFC multiplex assay also showed high diagnostic sensitivity and provides additional information on possible geographic origin.
Melioidosis is an emerging infectious disease caused by the Gram-negative soil bacterium *Burkholderia pseudomallei* (13). Infection is usually via cutaneous inoculation or inhalation and disease presentations range from asymptomatic, to localised skin infection or pneumonia, to disseminated disease with abscesses in multiple organs, resulting in fulminant sepsis with mortality rates > 50% (9). Increasing case numbers are being observed globally, likely reflecting both improved diagnostics (12) and a true increase in cases in those living in or travelling from endemic regions (11, 16, 17). Culture remains the gold standard for diagnosis of melioidosis, but is problematic due to sensitivity issues, lack of familiarity with *B. pseudomallei* in non-endemic laboratories (17) and poor specificity of biochemical tests (18). Subsequent delayed diagnosis can result in life-threatening delays in appropriate antimicrobial therapy (9).

Other diagnostic techniques for *B. pseudomallei* detection include antigen detection by immunofluorescence microscopy (34) or latex agglutination (3), however these suffer from reduced sensitivity or dependence on an initial culture step, delaying time to diagnosis (1). Culture is also required for MALDI-TOF mass spectrometry (14). Serological diagnosis is unreliable due to background antibody levels in endemic areas and low sensitivity and specificity (10, 33). While high-throughput sequencing technologies are not yet feasible for routine diagnostics (23), various other molecular platforms have been developed for rapid identification of *B. pseudomallei*. These include DNA microarrays (25), gene sequencing (15, 32), isothermal DNA amplification (7), and real-time PCR assays targeting specific regions of the *B. pseudomallei* genome (2, 5, 19-21, 26-30) (see Supplement Table).

Despite this abundance of published assays, the techniques used for validating criteria vary substantially between studies. Further, few have been evaluated directly on clinical samples (8, 20,
The aim of this study was to focus on real-time TaqMan PCR assays and assess the best available genomic target to date for *B. pseudomallei* detection in clinical samples. Seven real-time PCR assays were directly compared by assessing their analytical and diagnostic specificity and sensitivity (4, 6).

Based on superior reported specificity and LoD (see Supplement Table), four previously published real-time TaqMan PCR assays were included, namely TTS1-orf2 (22) and TTS1-orf11 (29) targeting the type III secretion (TTS) system gene cluster, *lpxO* (19) and 8653 (27) (see Table 1). The *mprA* target based on a previously published PCR assay (21) was validated with a TaqMan probe (Primer Express 3.0 software (Life Technologies)). A multiplex TaqMan assay targeting the *Yersinia*-like fimbrial / *Burkholderia thailandensis*-like flagellum and chemotaxis YLF/BTFC region (31) was also assessed, together with a newly developed dual-probe assay 266152 which targets the methylmalonate-semialdehyde dehydrogenase locus and differentiates between *B. pseudomallei* and *B. thailandensis* (see Table 1).

For analytical sensitivity and specificity, real-time PCR was carried out as previously reported (5). In brief, PCRs consisted of 10µl of 900 nM of primers, 200 nM of probe, 1X Applied Biosystems Genotyping Mastermix (Life Technologies), and 0.5 ng template DNA. Thermal cycling was performed on an AB 7900HT sequence detection system (Life Technologies) with 50°C 2 min, 95°C 10 min, 40 cycles of 95°C 15 s and 58°C 1 min. The 266152 assay was performed with 1X Applied Biosystems Universal Mastermix and 300 nM of primers. LoD was defined as the lowest possible template concentration detectable with 95% probability with at least 61/64 positive replicates (4, 6).

Specificity was evaluated by screening 365 *B. pseudomallei* strains and 115 non-*B. pseudomallei*
strains (with 71 Burkholderia spp. and 44 bacterial species of differential diagnostic importance (see full list in Supplement)). Ct value 40 was the declared cut-off for a positive result.

Clinical evaluation was performed by screening each assay across 50 clinical specimens (22 sputum, 20 blood and 8 urine samples) from 22 patients with acute melioidosis, 59% of whom presented with pneumonia and 18% with genitourinary infection, with 55% overall being bacteremic. These samples were part of a study comparing DNA extraction techniques for molecular detection of B. pseudomallei in clinical specimens (24); While all samples were from culture-confirmed melioidosis cases, not every specimen from each patient was cultured for B. pseudomallei. Blood samples from 22 healthy volunteers were used as negative controls. DNA was extracted using QIAamp DNA mini kit (Qiagen, Australia) and PureGene Blood Core Kit B (Qiagen). PCR conditions were as previously described (24) using the 1x Applied Biosystems Environmental Mastermix. Samples were declared positive if 2/2 duplicates had Ct values <40.

All assays showed high analytical specificity, with the TTS1_orf2, 8653, mprA and 266152 assays being 100% for both B. pseudomallei and non-target strains (see Table 2). The lpxO assay showed reduced specificity as it also amplified 14/23 Burkholderia mallei DNA targets. The TTS1_orf2 and YLF/BTFC assays had the lowest LoD of 5 genome equivalents (GE) per reaction (see Table 2). Variations to previously reported LoD were apparent from our data and are likely due to the strict LoD definition we used to determine the lowest possible template concentration detectable with 95% probability (4, 6). The low LoD of TTS1_orf2 and YLF/BTFC assays was also reflected in their high diagnostic sensitivity. Of the 43 clinical samples that tested positive for B. pseudomallei by one or more assays, 42 were positive by several assays and one only by TTS1_orf2. The mprA assay performed least well in the clinical evaluation and had a significantly lower detection rate than the TTS1_orf2, YLF/BTFC and lpxO assays (McNemar’s test for paired samples, P<0.001 for all, 2-tailed)(see Table 2).
These data support the TTS1_orf2 assay as the best performing assay to date for direct detection of
B. pseudomallei in clinical specimens. The multiplex assay YLF/BTFC also performed well and in
addition to B. pseudomallei detection, provides information on the potential geographic origin of the
tested isolate, with BTFC being common in Australia (88 %) but rare in Thailand (2 %) (31). As the YLF
locus was also found in some close relatives of B. pseudomallei (A. Tuanyok, unpublished data), this
assay should be used with caution on environmental samples.

As an additional informative assay, we included a dual-probe assay discriminating between B.
pseudomallei and B. thailandensis. This assay was designed for screening culture isolates and
showed high specificity. No differences in clinical detection rates were found when including both or
only the B. pseudomallei specific probe. Due to cross-hybridization, this assay should be used with
cautions on environmental samples where potentially both, B. pseudomallei and B. thailandensis
could be present.

Although the probe chemistry was tested as in the original publications, the PCR conditions were not
adjusted to the original optimized conditions for each assay; we used an adaptation of the TaqMan
Universal PCR Protocol (Life Technologies), with the same conditions for all assays. The inferior
performance of the mprA assay in the clinical evaluation may reflect that the original assay was not
probe-based and had an annealing temperature of 68°C, which differs from the conditions we used.

In summary, we have shown that the TTS1_orf2 assay provides the best available molecular target to
date for B. pseudomallei detection directly from clinical samples. Furthermore, the YLF/BTFC
multiplex assay which provides additional information on possible geographic origin of a B.
pseudomallei isolate also showed high diagnostic sensitivity.
We would like to thank the Microbiology Laboratory and medical and nursing colleagues at Royal Darwin Hospital for assistance in sample collection, and Alex Humphrey and Eleanor Woolveridge for laboratory assistance. We are thankful to the patients and volunteers who consented to providing clinical specimen. Ethics approval was granted by the Human Research Ethics Committee of the Northern Territory Department of Health and the Menzies School of Health Research (HREC 04/09). This project was funded by the Australian National Health and Medical Research Council (Project Grant 605820) and grants NIH NIAID U01-A1075568 and NIH NIAID U54-65359 and the US Department of Homeland Security (HSHQDC-10-C-00139).
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  Microbiology 42:3435-7
Table 1
Overview of primer and probe sequences of TaqMan real-time PCR assays validated in this report.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>Probe</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTS1-orf2 (115 bp)</td>
<td>CGTCCTCTATACTG</td>
<td>CGTGCACACCG</td>
<td>FAM-</td>
<td>(22)</td>
</tr>
<tr>
<td></td>
<td>TCGAGCAATCG</td>
<td>GTCAGTATC</td>
<td>CCGGAATCTGGATCACCACCACCTTTCC-BHQ</td>
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<tr>
<td>TTS1-orf11 (110 bp)</td>
<td>ATCGCCAATGCC</td>
<td>CAAATGGCCATC</td>
<td>FAM-TGGCGGAACCGGATTTGATCATC-TAMRA</td>
<td></td>
</tr>
<tr>
<td>lpxO (91 bp)</td>
<td>GGTTCCT</td>
<td>CGACGCCGCC</td>
<td>FAM-</td>
<td>TAMRA</td>
</tr>
<tr>
<td></td>
<td>TGTGTTTGCCCTAT</td>
<td>ACGTGCCGAACCGCCTATATC-G</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GCGTTCTC</td>
<td>GAGGAT</td>
<td>ACGTGCCGAACCGCCTATATC-G-BHQ</td>
<td></td>
</tr>
<tr>
<td>8653 (81 bp)</td>
<td>AGTCAAG</td>
<td>GTTCAAG</td>
<td>FAM-CGCGGAAGACCGCATC-GATC-TAMRA</td>
<td></td>
</tr>
<tr>
<td>mprA (199 bp)</td>
<td>ACTGCTTCGTTCA</td>
<td>TGACGGGCCCTGA</td>
<td>FAM-CAACTTGCAGTCAACTGA-MGB</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AGGCCGCCGTG</td>
<td>AGCGCCGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YLF/BTFC YLF (54 bp)</td>
<td>TGTGCTCGGCTCCA</td>
<td>CGTCCAGGGTCC</td>
<td>FAM-TGGCGGCTGCC-MGBNFQ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GATCAG</td>
<td>CGTTAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GCAATCCAATTCG</td>
<td>VIC-TGGCGGCTGGAAACA-MGB</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CGTCTCTAG</td>
<td>TTCCCTGTTGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>266152 (68 bp)</td>
<td>AATATACTAATA</td>
<td>AATAATCATAA</td>
<td>VIC-CGGTCTACACGCATGA-MGB</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCGAGGCCGGA</td>
<td>GACCGACATCA</td>
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<tr>
<td></td>
<td>GATGT</td>
<td>CAGCACG</td>
<td>FAM-CGGTCTACACGCAGCA-MGB</td>
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</tbody>
</table>

1“B.p” B. pseudomallei, “B.t” B. thailandensis

Price et al., manuscript in prep.)
Table 2
List of validation results of real-time PCR assays.

<table>
<thead>
<tr>
<th></th>
<th>TTS1orf2</th>
<th>TTS1orf11</th>
<th>lpxO</th>
<th>8653</th>
<th>mprA</th>
<th>YLF/BTFC</th>
<th>266152</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Analytical Specificity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B.p strains</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>99.7%</td>
<td>100%</td>
</tr>
<tr>
<td>Non-target strains</td>
<td>100%</td>
<td>99%</td>
<td>87.8%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td><strong>LoD (GE/rxn)</strong></td>
<td>5</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>50</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Diagnostic sensitivity</strong></td>
<td>80%</td>
<td>70%</td>
<td>76%</td>
<td>68%</td>
<td>54%</td>
<td>80%</td>
<td>68%</td>
</tr>
<tr>
<td>B.p strains</td>
<td>(40/50)</td>
<td>(35/50)</td>
<td>(38/50)</td>
<td>(34/50)</td>
<td>(27/50)</td>
<td>(40/50)</td>
<td>(34/50)</td>
</tr>
<tr>
<td>Non-target strains</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>95.5%</td>
<td>100%</td>
</tr>
</tbody>
</table>

1 The TTS1orf11 assay detected one of the *B. pseudomallei* strains only at a high Ct value of 36.9.
2 The *lpxO* assay provided false positive results for 14 / 23 *B. mallei* strains.
3 The TTS1orf11 assay provided a weak false positive result for 1 / 23 *B. mallei* strains at a Ct value of 37.4.
4 Number of *B. pseudomallei* genome equivalents per PCR. One *B. pseudomallei* genome equivalent (GE) equals approx. 7.8 fg based on a genome size of 7.2 Mb.
5 This LoD refers to a *B. pseudomallei* genome containing the YLF locus.
6 This likely reflects a contamination event as the weak false positive result (Ct value 37.7) was from a buffy coat sample of a healthy volunteer and weak positive results above the cut-off of 40 Ct values were also evident for this sample with other assays.