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


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Melioidosis is an emerging infectious disease caused by the soil bacterium Burkholderia pseudomallei. In diagnostic and forensic settings, molecular detection assays need not only 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 to be superior in detecting B. pseudomallei directly from clinical specimens. The YLF/BTFC multiplex assay (targeting the Yersinia-like fimbrial/Burkholderia thailandensis-like flagellum and chemotaxis region) 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 localized skin infection or pneumonia, to disseminated disease with abscesses in multiple organs, resulting in fulminant sepsis with mortality rates of >50% (9). Increasing numbers of cases are being observed globally, likely reflecting both improved diagnostics (12) and a true increase in cases in those living in or traveling from regions where melioidosis is endemic (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 laboratories in areas where the disease is nonendemic (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 matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (14). Serological diagnosis is unreliable due to background antibody levels in areas of endemicity 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 Table S1 in the supplemental material).

Despite this abundance of published assays, the techniques used for validating criteria vary substantially between studies. Furthermore, few have been evaluated directly on clinical samples (8, 20, 27, 28). Thus, it is difficult to determine which of these assays would perform best in a diagnostic or forensic setting, in which high specificity and sensitivity with a low limit of detection (LoD) are paramount.

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 specificities and sensitivities (4, 6).

Based on superior reported specificity and LoD (see Table S1 in the supplemental material), 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) (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 (Table 1).

For analytical sensitivity and specificity, real-time PCR was carried out as previously reported (5). In brief, PCR mixtures consisted of 10 μl of 900 nM primers, 200 nM probe, 1× Applied Biosystems genotyping master mix (Life Technologies), and 0.5 ng template DNA. Thermal cycling was performed on an AB 7900HT sequence detection system (Life Technologies) at 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 58°C for 1 min. The 266152 assay was performed with 1× Applied Biosystems genotyping master mix (Life Technologies). Thermal cycling was performed on an AB 7900HT sequence detection system (Life Technologies) at 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 58°C for 1 min. The 266152 assay was performed with 1× Applied Biosystems genotyping master mix (Life Technologies).
**TABLE 1** Overview of primer and probe sequences of TaqMan real-time PCR assays validated in this article

<table>
<thead>
<tr>
<th>Sequence of:</th>
<th>Reference</th>
<th>Forward primer (5'→3')</th>
<th>Reverse primer (5'→3')</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTS1-orf2 (115)</td>
<td>CGCTGCAACGCGCTAGATGGT</td>
<td>ATCGCCAAATGCCGGGTTTC</td>
<td>FAM-TCGGCGAACGCGATTTGATCGTTC-TAMRA</td>
<td>29</td>
</tr>
<tr>
<td>lpxO (91)</td>
<td>TTGTTTCGCCTATGCGTTCTC</td>
<td>CCACTCGCGCTTGAGGAT</td>
<td>FAM-ACGTGCCGAACACGCCGTATATCG-BHQ</td>
<td>19</td>
</tr>
<tr>
<td>8653 (81)</td>
<td>ATCGAATCAGGGCGTTCAAG</td>
<td>CATTCGGTGACGACACGACC</td>
<td>FAM-GCCGCAAGACGCCATCGTTCAT-TAMRA</td>
<td>27</td>
</tr>
<tr>
<td>YLF (54)</td>
<td>ATCGTGCCTTCCAGATCAG</td>
<td>CGGTCAGTTGCCCGCTATT</td>
<td>VIC-TCGGACCGCTTGCA-MGBNFQ</td>
<td>21</td>
</tr>
<tr>
<td>mprA and BTFC (96)</td>
<td>GGCAGCGTCGAACTGTTCTAG</td>
<td>CGAATCAATTCGTTTCCCTTGT</td>
<td>VIC-TTCGGCTGCGAAACA-MGBNFQ</td>
<td>23</td>
</tr>
<tr>
<td>B. pseudomallei</td>
<td>AATAAATCATAAACGTGAGGCC</td>
<td>AATAAATCATAAGACCGACATC</td>
<td>VIC-CGGTCTACACGCATGA-MGB</td>
<td></td>
</tr>
<tr>
<td>B. thailandensis (C)</td>
<td>FAM-CGGTCTACACGCACGA-MGB</td>
<td>GGAGATGTACGCACAGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; BHQ, black hole quencher.

Optimised conditions for each assay; we used an adaptation of the 1× Applied Biosystems environmental master mix. Samples were declared positive if 2/2 duplicates had Ct values of <40.

All assays showed high analytical specificity, with the TTS1-orf2, 8653, mprA and 266152 assays being 100% specific for both *B. pseudomallei* and nontarget strains (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 (Table 2). Variations from 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) (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 YLF/BTFC multiplex assay 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*, 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*, 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*, 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*, 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*, 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*, 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*, 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*, 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*, 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*, 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*, 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*, 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*, 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*, 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*, 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*, 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*, 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*, 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*, 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*.
TABLE 2: List of validation results from real-time PCR assays

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result by assay</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Analytical specificity</strong></td>
<td></td>
</tr>
<tr>
<td>B. pseudomallei strains</td>
<td></td>
</tr>
<tr>
<td>Analytical specificity, % (no. positive/total)</td>
<td>100 (365/365)</td>
</tr>
<tr>
<td>Non-target strains</td>
<td></td>
</tr>
<tr>
<td>Analytical specificity, % (no. positive/total)</td>
<td>100 (0/115)</td>
</tr>
<tr>
<td><strong>LoD (GE/reaction)</strong></td>
<td></td>
</tr>
<tr>
<td>Diagnostic sensitivity, % (no. positive/total)</td>
<td>99 (1/115)</td>
</tr>
<tr>
<td>Diagnostic specificity, % (no. positive/total)</td>
<td>87.8 (14/115)</td>
</tr>
</tbody>
</table>

a The TTS1-orf1 assay detected one of the B. pseudomallei strains only at a high C\(_T\) value of 36.9.
b The TTS1-orf1 assay provided a weak false-positive result for 1/23 B. mallei strains at a C\(_T\) value of 37.4.
c The lpxO assay provided false positive results for 14/23 B. mallei strains.
d Shown is the number of B. pseudomallei genome equivalents (GE) per PCR. One B. pseudomallei genome equivalent equals approximately 7.8 fg based on a genome size of 7.2 Mb.
e This LoD refers to a B. pseudomallei genome containing the YLF locus.

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-orf1 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 the possible geographic origin of a B. pseudomallei isolate, also showed high diagnostic sensitivity.

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