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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

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TABLE 1 Overview of primer and probe sequences of TaqMan real-time PCR assays validated in this article

Assay (size in bp)	Sequence of:		Probe[a]	Reference
	Forward primer (5′→3′)	Reverse primer (5′→3′)		
TTS1-*orf2* (115)	CGTCTCTATACTGTCGAGCAATCG	CGTGCACACCGGTCAGTATC	FAM-CCGGAATCTGGATCACCACCACTTTCC-BHQ	22
TTS1-*orf11* (110)	ATCGCCAAATGCCGGGTTTC	CAAATGGCCATCGTGATGTTC	FAM-TCGGCGAACGCGATTTGATCGTTC-TAMRA	29
lpxO (91)	TTGTTTCGCCTATGCGTTCTC	CCACTCGCGCTTGAGGAT	FAM-ACGTGCCGAACACGCCGTATATCG-BHQ	19
8653 (81)	ATCGAATCAGGGCGTTCAAG	CATTCGGTGACGACACGACC	FAM-CGCCGCAAGACGCCATCGTTCAT-TAMRA	27
mprA (199)	ACTGCTTCGTTCAAGGCGACCGT	TGACGGCCTGAACGTCCGC	FAM-CAACTTGACGATCAACTGA-MGB	21; this article
YLF/BTFC				31; this article
YLF (54)	TGCTCGGCTTCCAGATCAG	CGGTCAGTTGCCCGCTATT	FAM-TCGGACCGCTTGCA-MGBNFQ	
BTFC (96)	GGCAGCGTCGAACTGTTCTAG	CGAATCAATTCGTTTCCCTTGT	VIC-TTCGGCTGCGAAACA-MGBNFQ	
266152 (68)				E. P. Price et al., unpublished data
T (*B. pseudomallei*)	AATAAATCATAAACGTGAGGCC GGAGATGT	AATAAATCATAAGACCGACATC ACGCACAGC	VIC-CGGTCTACACGCATGA-MGB	
C (*B. thailandensis*)			FAM-CGGTCTACACGCACGA-MGB	

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

Biosystems universal master mix and 300 nM primers. The 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 the supplemental material]). A threshold cycle (C_T) value of 40 was the declared cutoff 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 the QIAamp DNA minikit (Qiagen, Australia) and PureGene blood core kit B (Qiagen). PCR conditions were as previously described (24), using the 1× Applied Biosystems environmental master mix. Samples were declared positive if 2/2 duplicates had C_T 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* (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 probes or only the *B. pseudomallei*-specific probe. Due to cross-hybridization, this assay should be used with caution 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

TABLE 2 List of validation results from real-time PCR assays

Parameter	Result by assay						
	TTS1-*orf2*	TTS1-*orf11*	*lpxO*	8653	*mprA*	YLF/BTFC	266152
Analytical specificity, % (no. positive/total)							
B. pseudomallei strains	100 (365/365)	100 (365/365)[a]	100 (365/365)	100 (365/365)	100 (365/365)	99.7 (364/365)	100 (365/365)
Nontarget strains	100 (0/115)	99 (1/115)[b]	87.8 (14/115)[c]	100 (0/115)	100 (0/115)	100 (0/115)	100 (0/115)
LoD (GE/reaction)[d]	5	10	10	10	50	5[e]	10
Diagnostic sensitivity, % (no. positive/total)	80 (40/50)	70 (35/50)	76 (38/50)	68 (34/50)	54 (27/50)	80 (40/50)	68 (34/50)
Diagnostic specificity, % (no. positive/total)	100 (0/22)	100 (0/22)	100 (0/22)	100 (0/22)	100 (0/22)	95.5 (1/22)[f]	100 (0/22)

[a] The TTS1-*orf11* assay detected one of the *B. pseudomallei* strains only at a high C_T value of 36.9.
[b] The TTS1-*orf11* 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.
[f] This likely reflects a contamination event as the weak false-positive result (C_T value of 37.7) was from a buffy coat sample from a healthy volunteer, and weak positive results above the cutoff of 40 C_T values were also evident for this sample with other assays.

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

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