Clinical Evaluation of a Type III Secretion System Real-Time PCR Assay for Diagnosing Melioidosis

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A *Burkholderia pseudomallei* type III secretion system real-time PCR assay was evaluated on clinical specimens in a region where melioidosis is endemic. The PCR was positive in 30/33 (91%) patients with culture-confirmed melioidosis. All six patients with melioidosis septic shock were blood PCR positive, suggesting potential for rapid diagnosis and commencement of appropriate therapy.

Melioidosis is the disease caused by infection with the environmental bacterium *Burkholderia pseudomallei*. In Southeast Asia and northern Australia, melioidosis is an important cause of community-acquired sepsis (1, 8). The clinical manifestations of melioidosis vary widely in terms of time course, severity, and organ system involvement, and it is difficult to differentiate from other causes of sepsis. In its most severe form, melioidosis progresses rapidly to septic shock, end organ failure, and death. The “gold standard” for diagnosis is culture from clinical samples, but isolation and identification of *B. pseudomallei* can take up to a week. Furthermore, *B. pseudomallei* is resistant to many antibiotics used in the empirical treatment of sepsis. A rapid diagnostic test would enable early therapy with appropriate antibiotics, potentially decreasing the mortality attributed to delayed therapy (8).

Serology is unreliable for early diagnosis due to both delayed or absent seroconversion and high background seropositivity in regions where melioidosis is endemic (2). Rapid immunofluorescence microscopy of sputum has shown excellent specificity but only 66% sensitivity (9). Various PCR tests for *B. pseudomallei* have been developed, but most of them have only been evaluated using pure bacterial cultures. Those evaluated on clinical samples from patients with suspected melioidosis had poor sensitivity and/or specificity (4, 5).

We initially evaluated a conventional PCR targeting a type III secretion system gene cluster (TTS1). This PCR demonstrated excellent specificity but was less sensitive than culture (3). We have subsequently converted the PCR to a real-time format (6), and we now report evaluation of the TTS1 real-time PCR on specimens collected from patients presenting with sepsis in an area where melioidosis is endemic.

Royal Darwin Hospital is a regional referral hospital located in the tropical north of Australia, where melioidosis is endemic. The study was approved by the Human Research Ethics

Committee of the Department of Health and Community Services and the Menzies School of Health Research. One hundred seven patients who presented with possible melioidosis had PCR performed on samples collected in parallel with those sent for culture. These included blood cultures, sputum, urine, pus, and other body fluids, as well as wound, throat, nose, and rectal swabs. Melioidosis was confirmed in 33 patients by culture of *B. pseudomallei* from one or more samples.

DNA was extracted from the clinical samples as previously described and was eluted in a volume of 200 μ l (3). Real-time PCR was performed using the Rotor-Gene 2000 (Corbett Research, Sydney, Australia). Samples were tested in duplicate using in each reaction 4 μ l of template and a final reaction volume of 25 μ l. The primers and fluorescent probe were as previously described (6). The final concentrations of the reagents were 0.42 μ M each primer, 0.26 μ M probe, 1 U HotStar Taq Polymerase (QIAGEN, Hilden, Germany), 0.2 mM deoxynucleotides, and 6.0 mM MgCl₂. The cycling parameters included an initial hold for 15 min at 95°C, 60 cycles of 15 s at 94°C and 60 s at 60°C, and a final hold for 2 min at 45°C. In each run, *B. pseudomallei*-positive and no-template controls were included.

Samples that were culture positive for *B. pseudomallei* and not real-time PCR positive by this method were retested in duplicate using a new protocol, which involved testing 23.5 μ l template in a reaction volume of 50 μ l. Sixteen blood samples from non-melioidosis patients were also tested in duplicate using this method. The methods were as described above, with the exceptions of MgCl₂ being increased to 6.2 mM and the denaturation time being increased to 30 s in each cycle.

Of the 33 patients with culture-confirmed melioidosis, 30 had one or more real-time PCR-positive samples, giving 91% sensitivity for patient diagnosis. Four of 74 non-melioidosis patients also had a real-time PCR-positive sample, giving specificity of 95%. These four patients all had respiratory infections which responded to a short course of antibiotics. None received specific melioidosis therapy or subsequently developed confirmed melioidosis.

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TABLE 1. Samples from 33 culture-confirmed melioidosis patients

Sample type	No. of samples	No. (%) of real-time PCR-positive samples	
		25- μ l protocol	25- μ l and 50- μ l protocols ^a
Blood			
Culture positive	55	20 (36)	31 (56)
Culture negative	23	4 (17)	
Sputum			
Culture positive	14	14 (100)	
Culture negative	8	8 (100)	
Throat swab			
Culture positive	9	6 (66)	6 (66)
Culture negative	16	3 (19)	
Rectal swab			
Culture positive	5	2 (40)	2 (40)
Culture negative	17	1 (6)	
Wound swab			
Culture positive	10	10 (100)	
Culture negative	1	0	
Nose swab			
Culture positive	2	2 (100)	
Culture negative	2	1 (50)	
Urine			
Culture positive	5	5 (100)	
Culture negative	11	8 (73)	
Prostatic drainage fluid			
Culture positive	5	5 (100)	
Culture negative	0	0	
Joint fluid			
Culture positive	4	3 (75)	4 (100)
Culture negative	1	0	
Pleural fluid			
Culture positive	1	1 (100)	
Culture negative	2	1 (50)	
Pericardial fluid			
Culture positive	0	0	
Culture negative	1	1 (100)	
Prostate tissue			
Culture positive	1	1 (100)	
Culture negative	0	0	
Ankle tissue			
Culture positive	1	1 (100)	
Culture negative	0	0	

^a Samples which were culture positive but real-time PCR negative using the 25- μ l reaction protocol were tested using the 50- μ l method.

Table 1 shows the culture and real-time PCR results of individual samples collected from melioidosis patients. On sputum, urine, drained pus, and wound swabs, the assay performed with 100% sensitivity compared to culture. The sensitivity of the assay on blood samples depended on the severity of clinical disease. Fourteen of 19 (74%) culture-positive blood samples from patients with septic shock were real-time PCR positive using the 25- μ l reaction protocol, compared to 6 of 36

TABLE 2. Samples from 74 non-melioidosis patients

Sample type	No. of samples	No. (%) of real-time PCR-positive samples, 25- μ l protocol
Blood	58	0
Sputum	28	0
Throat swab	49	3 (6)
Rectal swab	47	1 (2)
Wound swab	5	0
Urine	3	0
Pleural fluid	2	0
Nose swab	7	0
Prostatic drainage	1	0
Drainage of bone collection	1	0
Pericardial fluid	1	0
Cerebrospinal fluid	1	0
Intervertebral disc aspirate	1	0
Pus	1	0

(17%) culture-positive blood samples from patients without septic shock ($P < 0.001$; Fisher exact test). All six patients with melioidosis bacteremia with septic shock had at least one blood PCR-positive result, compared with only 4/14 patients with bacteremia without septic shock ($P = 0.005$; Fisher exact test). When the culture-positive, PCR-negative blood samples were tested using the 50- μ l method, 11/35 were positive.

Table 2 shows the real-time PCR results for non-melioidosis patient samples. Four of 205 samples were real-time PCR positive. In addition, 1 of 16 blood samples from non-melioidosis patients tested at the 50- μ l reaction volume was real-time PCR positive. At no point were the no-template controls at either reaction volume positive.

The TTS1 real-time PCR assay performed with 100% sensitivity and specificity on sputum, urine, drained pus, and wound swabs, with the high sensitivity reflecting the high bacterial load in samples from localized sites of infection. The PCR was less sensitive on blood samples. However, for the samples from those patients with melioidosis septic shock the real-time PCR result was usually positive, and this is the patient cohort for whom a rapid diagnosis may be critical for survival. Blood cultures involved four 10-ml samples, while only 4 ml EDTA blood was collected for real-time PCR. Furthermore, the DNA was only extracted from the buffy coat, with only 4 μ l of the 200 μ l eluted DNA being tested in each reaction. The increase in sensitivity associated with septic shock and also with the 50- μ l method suggests small bacterial numbers in negative samples. This is consistent with a previous report that 45% of septicemic melioidosis patients have <1 CFU/ml in blood (7).

Sensitivity for blood samples could potentially be improved by DNA extraction from a larger volume of sample and elution in a smaller volume of buffer. Multiple PCR replicates could also increase the likelihood of obtaining a positive result for those samples with a DNA concentration at the lower limit of detection. It is also possible that PCR inhibitors such as heme and human DNA contribute to the lower sensitivity of blood samples. A real-time PCR for diagnosing brucellosis was more sensitive on serum than whole blood (10). On preliminary further sampling of melioidosis patients, we have also found pellets from centrifuged plasma to be more sensitive than the

buffy coat. For blood samples, we now elute DNA in 100 μ l and test 8 μ l of template in a final reaction volume of 25 μ l.

Some culture-negative but real-time PCR-positive samples were obtained from melioidosis patients. This probably usually represents the detection of nonviable bacteria, as the majority of these culture-negative but PCR-positive samples were taken following commencement of specific melioidosis therapy. It is unclear what the false-positive results from non-melioidosis patients represent, but possibilities include contamination, cross-reactivity with an alternative DNA sequence, detection of undiagnosed melioidosis, or asymptomatic *B. pseudomallei* carriage. The false-positive reactions were from swabs from mucosal surfaces, and we currently recommend using this real-time PCR only for blood, sputum, urine, cerebrospinal fluid, and pus samples.

In summary, the TTS1 real-time PCR assay showed sensitive and specific detection of *B. pseudomallei* DNA in samples from localized sites of infection. Sensitivity in blood samples was improved over our previous conventional PCR (3), and the assay has the potential to make a rapid diagnosis in patients with melioidosis septic shock. Further evaluation is required in other locations where melioidosis is endemic, with testing of samples from multiple tissue sites in parallel with culture.

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