Am. J. Trop. Med. Hyg., 73(6), 2005, pp. 1162–1164

SHORT REPORT: APPLICATION OF A POLYMERASE CHAIN REACTION TO DETECT *BURKHOLDERIA PSEUDOMALLEI* IN CLINICAL SPECIMENS FROM PATIENTS WITH SUSPECTED MELIOIDOSIS

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Abstract. The diagnostic potential of a *Burkholderia pseudomallei* type three secretion system (TTS1) polymerase chain reaction (PCR) was examined on clinical specimens from 27 patients with sepsis in the Northern Territory of Australia, a region endemic for melioidosis. The TTS1 PCR was conducted on DNA extracted from a range of clinical specimens (blood, sputum, urine, joint, pericardial and pleural fluid, and swabs from skin lesions, throat, nose, and rectum). The PCR sensitivity in culture-positive clinical specimens from the nine confirmed patients with melioidosis was 65% and the specificity was 100%, with no PCR-positive results in specimens from 18 patients without melioidosis. The PCR based on the *B. pseudomallei* TTS1 has the potential to substantially improve the timeliness of diagnosis of melioidosis.

Burkholderia pseudomallei is a soil and water bacterium that is responsible for melioidosis, a life-threatening disease endemic in northern Australia and southeast Asia.[1,2] The diagnosis of melioidosis can be difficult and delayed therapy is associated with higher mortality.[3] Culturing clinical specimens is the gold standard for diagnosis, but isolating and identifying *B. pseudomallei* takes at least 2–7 days.[4] Because *B. pseudomallei* is resistant to many antibiotics used in empirical therapy of sepsis, more rapid diagnosis of melioidosis would enable earlier institution of appropriate treatment.

A number of polymerase chain reactions (PCRs) have been developed for identification of *B. pseudomallei.* The PCR targets have included 23S ribosomal RNA (rRNA),[5] 16S rRNA,[6] the 16S–23S rRNA intergenic region,[7] LPS,[8] and flagellin C (*fliC*) and ribosomal protein subunit S21 (*rpsU*).[9] Sensitivity and specificity of these PCRs for *B. pseudomallei* have generally been excellent when testing bacterial cultures. However there have been problems with both sensitivity[10] and specificity[10–12] on the application of these PCRs for diagnosis directly in patient clinical specimens.

We have investigated a PCR based on the type three secretion (TTS1) system gene cluster of *B. pseudomallei*.[13,14] This PCR had a sensitivity and specificity of 100% for *B. pseudomallei* when tested on a range of bacterial isolates of *B. pseudomallei* and other species.[15] We present data on its utility for diagnosis of melioidosis using clinical samples.

Royal Darwin Hospital is in the tropical north of the Northern Territory of Australia, where melioidosis is an important cause of community-acquired sepsis.[2] Specimens were collected from 27 patients admitted to Royal Darwin Hospital with sepsis where melioidosis was included in the initial differential diagnosis. Duplicates of samples sent for culture to the microbiology laboratory were collected for PCR.

Genomic DNA was extracted from clinical specimens using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Samples (200 μL) were used for sputum, body fluids and buffy coat from blood samples stored in EDTA. For swabs, a lysate was used after hydration with normal saline.

The oligonucleotide primers used to amplify a 548-basepair region of the *B. pseudomallei* TTS1 gene cluster, BPTTSF and BPTTSR, were as previously described.[14] The PCR was conducted in a final reaction volume of 25 μL containing 1 μL of template DNA, 1 unit of *Taq* DNA polymerase (Qiagen), 1× PCR buffer (Qiagen), 200 μM of deoxynucleotides dATP, dCTP, dGTP, and dTTP (Roche, Basel, Switzerland), and 500 nM of each oligonucleotide primer. The PCR was performed on a Palm-Cycler (Corbett Research, Sydney, New South Wales, Australia) with an initial denaturing step at 95°C for 1 minute; an extension step at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds for 35 cycles; and a final extension step at 72°C for 2 minutes. Samples were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized with the Gel Doc 1000 gel documentation system (Bio-Rad Laboratories, Hercules, CA).

To improve sensitivity, the DNA from clinical specimens that were culture positive for *B. pseudomallei* but PCR negative was subjected to an additional PCR. In this second TTS1 PCR, the amount of template DNA tested was increased from 1 μL to 5 μL, the initial denaturing step at 95°C was extended to 4.5 minutes, and the PCR was increased to 40 cycles.

This study was reviewed and approved by the Human Research Ethics Committee of Territory Health Services and the Menzies School of Health Research.

Of the 27 patients, 9 had melioidosis confirmed by culture of *B. pseudomallei.* The other 18 patients were culture negative for *B. pseudomallei* and none was treated for melioidosis or developed melioidosis over the next 18 months. In the melioidosis patients, 1 μL of DNA in the TTS1 PCR had a sensitivity of 46%, with 12 of 26 culture-positive specimens being PCR positive (Table 1). When the 14 PCR negative/culture positive specimens were retested using 5 μL of DNA, an additional 5 were PCR positive, giving an overall sensitivity of 65%. All 51 specimens from the 18 patients without melioidosis were PCR negative (Table 1), indicating a PCR assay specificity of 100% with these clinical specimens.

A range of clinical specimens was tested (Table 2). Although the PCR with blood buffy coat was positive in only two of seven concomitant positive blood culture samples, all seven culture-positive sputum samples were PCR positive.

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TABLE 1

Burkholderia pseudomallei type three secretion system 1 (TTS1) polymerase chain reaction (PCR) results on clinical specimens

	First TTS1 PCR*		First TTS1 + second TTS1 PCR†	
	PCR Positive	PCR Negative	PCR Positive	PCR Negative
Melioidosis patients (n = 9)				
Culture-positive specimens (n = 26)	12	14	17	9
Culture-negative specimens (n = 16)	3	13	–	–
Non-melioidosis patients (n = 18)	0	51	–	–

* PCR using 1 μL of template DNA.
† PCR using 5 μL of template DNA. This second PCR was only performed on culture-positive specimens where the 1 μL template DNA was negative.

Furthermore, two sputum samples collected after the start of treatment with antibiotics were culture negative but PCR positive (Table 2). The only other PCR-positive/culture-negative sample was a nose swab from a bacteremic melioidosis patient. The PCR successfully identified infection in joints, skin, and pericardium. Although multiple specimens were taken from each melioidosis patient, the PCR failed to diagnose two of the nine melioidosis cases.

As with other PCRs, the TTS1 PCR has previously been shown to be sensitive and specific in identifying *B. pseudomallei* from DNA extractions of pure cultures.[15] However, when other PCRs were tested on patient clinical specimens, false-positive results have occurred in patients without melioidosis, including results in our laboratory.[10,11] Because of the nature and duration of antibiotics required for therapy of melioidosis,[1] it is critical that any rapid diagnostic test proposed for the diagnosis of melioidosis as an alternative to culture of *B. pseudomallei* has a specificity of 100%.

Although the lack of false-positive results in this study is encouraging, the sensitivity of the TTS1 PCR requires improvement. The PCR of clinical specimens requires detection of bacterial DNA in the presence of potential PCR inhibitors and significantly high concentrations of patient DNA. Improvement in sensitivity occurred when the template DNA from the specimen was increased from 1 μL to 5 μL. The total (human plus bacterial) DNA in the 5 μL template ranged from 225 ng to 1,475 ng in sputum samples and from 75 ng to 250 ng in buffy coat samples.

TABLE 2

Results by specimen type from melioidosis patients*

Specimen	TTS1 PCR		Culture	
	Positive	Negative	Positive	Negative
Blood (n = 8)	2	6	7	1
Sputum (n = 9)	9	0	7	2
Throat swab (n = 7)	2	5	4	3
Nose swab (n = 4)	2	2	2	2
Rectal swab (n = 6)	0	6	0	6
Knee joint aspirate (n = 2)	1	1	2	0
Pericardial aspirate	1	0	1	0
Pleural aspirate	0	1	0	1
Ankle joint aspirate	1	0	1	0
Urine	0	1	0	1
Skin abscess swab	1	0	1	0
Skin pustule swab	1	0	1	0

* For definitions of abbreviations, see Table 1.

The TTS1 PCR is robust for sputum samples, which reflects the high bacterial load in sputum. The ability of this PCR to detect presumably dead or non-viable *B. pseudomallei* in culture-negative sputum from melioidosis patients already taking antibiotics suggests a potentially useful role in diagnosis where treatment with antibiotics has begun before adequate cultures have been obtained. It is also encouraging that the PCR successfully identified infection in joints, skin, and pericardium.

Quantitative blood culture studies have shown that even in septicemic melioidosis the number of bacteria in the blood may be small, with 45% of samples containing less than one colony-forming unit per milliliter of blood in one study.[16] This may explain the low sensitivity of PCR in blood buffy coat that we found, with both PCR-positive samples only being detected with the 5 μL DNA in the PCR. As well as the small sampling volume, PCR inhibitors such as heme may remain after blood sample processing. Testing larger sample volumes and multiple-sample PCRs are options for improving the sensitivity of the buffy coat PCR.

In summary, although the 100% specificity of the *B. pseudomallei* TTS1 PCR in clinical specimens to date is promising, improvements in sensitivity are required, especially for blood samples. The detection sensitivity may be improved by adapting the current TTS1 PCR into a real-time PCR, as has recently been done for several other *B. pseudomallei* PCR targets.[9] However, it will be critical to evaluate any new real-time PCRs on adequate numbers of clinical specimens from patients both with and without melioidosis. In the meantime, culture of *B. pseudomallei* remains the gold standard for diagnosis of melioidosis.

Received June 24, 2005. Accepted for publication August 10, 2005.

Acknowledgments: We are grateful to Dr. Gary Lum and the microbiology staff at Royal Darwin Hospital Pathology for culturing clinical specimens and identifying the bacteria. We also thank our clinical colleagues at Royal Darwin Hospital for their assistance in collecting patient specimens and Dr. Jay Gee (Meningitis and Special Pathogens Branch, Centers for Disease Control and Prevention, Atlanta, Georgia) for providing the Qiagen kits.

Financial support: This study was supported by a Project Grant from the Australian National Health and Medical Research Council.

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