Prospective Clinical Evaluation of the Accuracy of 16S rRNA Real-Time PCR Assay for the Diagnosis of Melioidosis

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Abstract. The accuracy of a Burkholderia pseudomallei 16s rRNA real-time PCR assay was evaluated against culture for the diagnosis of melioidosis in Thailand. A total of 846 samples were obtained from 383 patients with suspected melioidosis. One or more specimens were PCR positive for 47 of 77 patients with culture-proven melioidosis (sensitivity 61.0%, 95% CI: 49.2–72.0%). PCR was negative for all 306 patients who were culture negative for B. pseudomallei (specificity 100%, 95% CI: 98.8–100%). Diagnostic sensitivity of PCR was 22.7% for patients who were culture positive for blood only, compared with 79.4% for patients who were culture positive for samples other than blood. The median (interquartile range) B. pseudomallei colony count in blood for 44 of 77 patients with positive blood cultures was 2.4 CFU/ml (0.2–13.5 CFU/ml); this may explain the low sensitivity of PCR for this specimen. The PCR assay described here is not sufficiently sensitive to replace culture in our clinical setting.

INTRODUCTION

Burkholderia pseudomallei is a Category B biothreat agent and the cause of melioidosis.1,2 Bacterial culture of B. pseudomallei represents the current gold standard for the diagnosis of this infection. Rapid detection of B. pseudomallei in clinical samples using latex agglutination3–5 or direct immunofluorescent microscopy6 has been described, but many of these reagents are not commercially available, some specimens are not suitable for testing, and their sensitivity is lower than culture. Serological diagnosis is a poor alternative in regions where B. pseudomallei exists in the soil because antibodies to the organism are commonly present in the healthy population from a young age.6–8 Real-time PCR targeting B. pseudomallei genes encoding 16s rRNA, flagellin (flaC), ribosomal protein subunit S21 (rpsU), and type III secretion system (TTS) genes have been evaluated using pure bacterial cultures.9–11 One real-time PCR assay targeting a gene in the TTS1 cluster has been evaluated in a clinical setting to date.12 Of 107 patients presenting with suspected melioidosis at the Royal Darwin Hospital, Northern Australia, 33 patients had culture-confirmed infection; PCR had a diagnostic sensitivity of 91%. The 16s rRNA gene target has a potential advantage over others because the B. pseudomallei K96243 genome contains 4 copies,13 and this may enhance assay sensitivity. The aim of this study was to undertake a large prospective clinical evaluation of a 16s rRNA real-time PCR assay in melioidosis-endemic northeast Thailand.

MATERIALS AND METHODS

Study population and sampling. A prospective study was conducted at Sappasithiprasong Hospital, Ubon Ratchathani, northeast Thailand between August 15 and September 30, 2006. This was approved by the Human Research Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok. Patients with suspected melioidosis were identified during twice daily ward rounds of the medical and intensive care wards. Multiple samples were taken from suspected cases for culture and PCR, including blood, throat swab, sputum/tracheal aspirate, urine, pus, or surface swab from wounds and skin lesions. A 20-ml blood sample was divided between an EDTA tube for PCR (5 ml), a BacT/ ALERT® FA bottle (BioMérieux, NC) for standard culture (5 ml), and an Isolator 10 lysis centrifugation tube (Oxoid, Basingstoke, Hampshire, UK) for quantitative count of B. pseudomallei (10 ml).14 Urine, pus, and respiratory secretions were placed into plain sterile containers, and swabs were transported to the laboratory dry. Specimens were cultured and B. pseudomallei identified as previously described.4,15,16 Direct immunofluorescent microscopy (DIF) was performed on sputum/respiratory secretions, urine, pus, and other body fluids; blood and swabs are not suitable for DIF.

PCR assay. DNA was extracted from clinical specimens within ~2 hours of collection. EDTA blood was spun at 1,500 × g for 10 minutes and the buffy coat removed using a Pasteur pipette, 200 μL of which was used for DNA extraction. Ten milliliters of urine was centrifuged at 1,500 × g for 5 minutes, and 9 mL of supernatant removed to obtain a 10 X concentrated urine; 200 μL of this was used for DNA extraction. Respiratory secretions were used neat unless highly viscous, when an equal volume of sterile distilled water was added. DNA was extracted directly from pus and other body fluids. Swabs were placed into 500 μL of sterile distilled water for 10 minutes and then vortexed for 1 minute. Using these preparations, 200 μL of each sample was transferred into a 1.5-mL reaction tube containing 200 μL lysis buffer and 20 μL of 20 mg/ml proteinase K. The mixture was vortexed and incubated in a water bath at 56°C with continuous shaking for 10 minutes. DNA was extracted using an automated DNA extractor (KingFisher ml, Labsystems) and the InviMag® Blood DNA Mini Kit/KF ml (KingFisher ml), as recommended by the manufacturer. DNA from blood was eluted in a volume of 100 μL, and DNA from other specimens was eluted in a volume of 200 μL.

Plasmid standards were constructed by cloning the 16s
rRNA fragment amplified from *B. pseudomallei* K96243 DNA into pGEM-T Easy Vector Systems (Promega, WI), as instructed by the manufacturer. DNA concentration was determined following linearization using the Quant-iT™ High-sensitivity DNA assay kit (Invitrogen, CA) and the Rotor-Gene 3000 using the DNA concentration measurement mode. A standard curve was constructed by plotting the logarithmic values of a known number of bacterial copies versus the cycle threshold (Ct) value. This revealed a linear assay over 6 orders of magnitude (6.5 to 6.5 x 10^9 target copies/reaction). The lower limit of the assay was 6 target copies/reaction (5 µl template). Four replicates from a 10-fold serial dilution of plasmid standard were assayed in a single run to determine intra-assay variation, and 4 replicates were assayed over 3 consecutive days to determine inter-assay variation. The inter-assay variation was 0.78–2.50%, and the intra-assay variation was 0.55–1.86%.

The PCR assay was performed on samples on the day of collection by a technician who was blinded to the culture result; PCR results were not revealed to clinicians or other laboratory staff. The PCR assay used fluorescent hybridization probes to amplify a region of the 16s rRNA gene as described previously, with the exception of an alternative reverse primer (5’ CAACAACTAGTTGACATCGTTTA-3’ (position 730-708, GenBank accession number AF093060)). The reverse primer was designed, and primers and probes were synthesized by TIB MOLBIOL (Berlin, Germany). The primer pair generated a 378-bp product. Reactions were carried out in a total volume of 20 µL using the Rotor-Gene 3000 real-time PCR machine (Corbett Robotics, Sydney, Australia). PCR mixtures contained primers and probes at final concentrations of 0.25 µM and 0.25 µM, respectively, 10 µL of quantiprobe (containing MgCl2, Taq DNA polymerase, deoxyribose triphosphates, and reaction buffer; Quantimix Easy Probes kit, Biotools B&M Laboratories, Madrid, Spain), 5 µL of template DNA, and 3 µL of nuclease-free distilled water. Cycling conditions were 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds, 57°C for 30 seconds, and 72°C for 20 seconds. Melt curve analysis was performed between 50°C and 80°C. Data were analyzed using the Rotor-Gene software version 6.0. A sample was considered positive for *B. pseudomallei* DNA if a signal was produced with a melting temperature (Tm) between 68.0 and 71.0. Each PCR run included negative controls (water) and positive plasmid controls. Quantitation of *B. pseudomallei* in blood samples was carried out by comparison with the standard curve.

**Statistical analyses.** All statistical analyses were performed using STATA/SE version 9.0 (College Station, TX). Standard bacterial culture was used to calculate the sensitivity and specificity of PCR. The χ² test or Fisher’s exact test were used to compare categorical variables between the different groups, and the McNemar test was used to compare the paired groups. The Kruskal-Wallis test was used to compare continuous data between groups.

**RESULTS**

A total of 846 samples were obtained from 383 patients with suspected melioidosis. Sample types were blood (N = 233), sputum (N = 192), urine (N = 197), throat swab (N = 176), surface swab from wounds or skin lesions (N = 16), and pus or other body fluids from sterile sites (N = 32). Melioidosis was diagnosed in 77 patients who were positive for *B. pseudomallei* in 116 samples.

A positive PCR result was obtained for one or more specimens in 47 of 77 patients (61%) with culture-confirmed melioidosis, a sensitivity for patient diagnosis of 61.0% (95% confidence interval (CI) 49.2–72.0%). All samples from 306 patients that were culture-negative for *B. pseudomallei* were negative by PCR (specificity 100%, 95% CI 98.8–100%). The positive predictive value was 100% (95% CI: 92.5–100%), negative predictive value was 91.1% (95% CI: 87.5–93.9%), and diagnostic accuracy was 92.2% (95% CI: 89.0–94.7%).

Twenty-one of 77 patients with melioidosis were culture-positive from blood and at least one other specimen type; the sensitivity of PCR for patient diagnosis in this group was 71.4% (95% CI: 47.8–88.7%). Thirty-four patients were culture-positive for samples other than blood; the sensitivity of PCR for patient diagnosis in this group was 79.4% (95% CI: 62.1–91.3%). The remaining 22 patients were culture-positive for blood only; diagnostic sensitivity of PCR for this group was 22.7% (95% CI: 7.8–45.4%). At the time of specimen collection, 19 of 77 (25%) culture-confirmed melioidosis patients had septic shock. PCR was positive in 11 (58%) of these patients.

An analysis was also performed using specimen type as the denominator. PCR was positive for 59 of 116 specimens that were culture-positive for *B. pseudomallei* (50.9%, 95% CI: 41.4–60.1%), and negative for 723 of 730 specimens that were culture-negative for *B. pseudomallei* (99.0%, 95% CI: 98.0–99.6%). All 7 samples that were PCR positive but culture negative for *B. pseudomallei* were taken from patients with culture-proven melioidosis (culture of samples taken from another body site were positive). It is likely that these samples contained non-viable *B. pseudomallei*. The sensitivity and specificity of PCR for each specimen type is shown in Table 1. PCR sensitivity was highest for pus and other body fluids (85.7%) and surface swabs (88.9%), but was only 25% for blood. PCR was repeated for all blood samples using a larger volume reaction mix containing 20 µL of template; this did not increase the sensitivity of the assay (data not shown).

The number of colony forming units/ml (CFU/ml) in blood was defined for 44 patients with positive blood cultures using

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**TABLE 1**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>PCR +ve</th>
<th>Sensitivity (95% CI)*</th>
<th>PCR +ve</th>
<th>Specificity (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>11/44</td>
<td>25.0%</td>
<td>0/189</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>(13.2–40.3%)</td>
<td>(98.1–100%)</td>
<td>(96.7–100%)</td>
<td>(94.3–99.9%)</td>
</tr>
<tr>
<td>Sputum</td>
<td>17/24</td>
<td>70.8%</td>
<td>1/168</td>
<td>99.4%</td>
</tr>
<tr>
<td></td>
<td>(48.9–87.4%)</td>
<td>(96.7–100%)</td>
<td></td>
<td>(97.4%)</td>
</tr>
<tr>
<td>Urine</td>
<td>6/8</td>
<td>75.0%</td>
<td>5/189</td>
<td>97.4%</td>
</tr>
<tr>
<td></td>
<td>(34.9–98.6%)</td>
<td>(94.3–99.9%)</td>
<td></td>
<td>(94.3–99.9%)</td>
</tr>
<tr>
<td>Pus, fluid collection</td>
<td>12/14</td>
<td>85.7%</td>
<td>1/18</td>
<td>94.4%</td>
</tr>
<tr>
<td></td>
<td>(57.2–98.2%)</td>
<td>(72.3–99.9%)</td>
<td></td>
<td>(94.3–99.9%)</td>
</tr>
<tr>
<td>Throat swab</td>
<td>5/17</td>
<td>29.4%</td>
<td>0/159</td>
<td>97.7%</td>
</tr>
<tr>
<td></td>
<td>(10.3–56.0%)</td>
<td>(97.7–100%)</td>
<td></td>
<td>(97.7–100%)</td>
</tr>
<tr>
<td>Other swab</td>
<td>8/9</td>
<td>88.9%</td>
<td>0/7</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>(51.8–99.7%)</td>
<td>(59.0–100%)</td>
<td></td>
<td>(98.0–99.6%)</td>
</tr>
<tr>
<td>Overall</td>
<td>59/116</td>
<td>50.9%</td>
<td>7/730</td>
<td>99.0%</td>
</tr>
<tr>
<td></td>
<td>(41.4–60.1%)</td>
<td>(98.0–99.6%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 95% confidence intervals.
parallel isolator tube culture. The *B. pseudomallei* count ranged from < 0.1 to >100 CFU/ml of blood; the median (interquartile range [IQR]) was 2.4 CFU/ml (0.2 to 13.5 CFU/ml). Of the 11 blood samples positive by PCR, only one sample with >100 CFU/ml on culture gave a quantitative PCR result (20 copies per reaction using 5 µL template); the remainder fell below the linear part of the standard curve. A positive PCR result for blood was associated with a higher colony count; the median (IQR) counts for PCR positive and negative samples were 22 CFU/ml (IQR 3.1–50 CFU/ml) and 0.7 CFU/ml (IQR 0.1–4.7 CFU/ml), respectively (*P* = 0.02).

Forty-eight patients with culture confirmed melioidosis were receiving effective parenteral antimicrobials at the time of sampling was performed (45 ceftazidime, 2 amoxicillin-clavulanic acid, and 1 cefoperazone plus sulbactam). This group had a lower bacterial count in blood (median 0.5 CFU/ml, IQR 0.1–7.7 CFU/ml) compared with patients not receiving antibiotics at the time of sampling (median 8.4 CFU/ml, IQR 0.8–61 CFU/ml) (*P* = 0.03). The overall mortality rate for patients with melioidosis during hospital admission was 34 of 77 (44%). The mortality of 11 patients with a positive PCR result for blood was 100%, compared with a mortality rate of 35% for the remainder (*P* < 0.001).

A total of 421 samples were suitable for DIF, of which 415 samples were examined (sputum *N* = 189, urine *N* = 196, and pus *N* = 30). Forty-three of the 415 samples were culture-positive for *B. pseudomallei*. DIF was positive in 19 of 43 (sensitivity 44.2%, 95% CI: 29.1–60.1%), and was negative for all samples that were culture-negative for *B. pseudomallei* (specificity 100%, 95% CI: 99.0–100%). PCR was positive in 33 of these 43 samples (sensitivity 76.7%, 95% CI: 61.4–88.2%) (*P* < 0.001, McNemar test).

Polymerase chain reaction was completed and the results available by the end of each working day. The time taken to extract DNA and complete the assay was ~3 hours. Bacterial agar plates were examined daily, and colonies were identified as *B. pseudomallei* on the same day using a highly sensitive and specific latex agglutination test, followed by conventional laboratory confirmation thereafer. Median times to identification of *B. pseudomallei* were 24 hours for sputum, urine, surface swab, and pus or other body fluid, 48 hours for blood, and 72 hours for throat swab.

**DISCUSSION**

Real-time PCR plays an increasing part in the rapid diagnosis of a range of infectious diseases. A previous evaluation of a real-time PCR assay targeting a gene in the TTS1 cluster conducted at the Royal Darwin Hospital in Australia indicated that PCR may have a role in the rapid diagnosis of this melioidosis. The findings reported here lead to a less optimistic interpretation; the overall sensitivity of 61% indicates that this assay is not sufficiently sensitive to replace culture in our setting.

Prior to commencing the study, we had predicted based on the Darwin study that the sensitivity of our assay would be lowest for blood. To evaluate whether low colony counts in blood could account for this finding, we used isolator tube cultures to determine the *B. pseudomallei* colony count in the same specimen. As predicted, the sensitivity of PCR for blood was low. The median *B. pseudomallei* colony in the blood of patients with positive blood cultures was very low, and was significantly lower for patients with a false negative PCR result for blood compared with patients who were PCR positive. Our finding of a low colony count in blood is consistent with previous reports; 53% of blood cultures contained <10 CFU/ml as defined by pour plate in one study, and 62% and 72% had ≤10 CFU/ml as defined by pour plate and isolator tube, respectively, in a second study.

An important contributor to the low *B. pseudomallei* count in blood was the number of patients already receiving effective antimicrobials by the time of sampling. These patients were not necessarily known to have melioidosis, and this practice reflects effective empirical prescribing for febrile patients in an area where melioidosis is common. We function as a melioidosis research team, and the patients are first seen, investigated, treated, and admitted by the attending physicians rather than by us. This may have a significant effect on the probability of a positive PCR result on samples collected some hours later. It is normally possible in clinical practice to obtain blood and other samples from patients on admission prior to antibiotic treatment without any delay to drug administration. We believe that this would increase the sensitivity of the PCR assay described here, a suggestion that merits evaluation.

The low sensitivity of PCR for blood using 4 µL template DNA reported in the Darwin study (36%, 95% CI: 23.8–50.4%), is similar to our findings. On comparison of protocols, we extracted the DNA from the same volume of buffy coat and used 5 µL of DNA template as compared with 4 µL. In the Darwin study, sensitivity of PCR for blood samples rose to 56% when template was increased from 4 µL to 23.5 µL. This was not reproduced here; we hypothesize that this may relate to increased amounts of inhibitor.

With the exception of throat swabs, the sensitivity of PCR for other specimens was considerably higher than that for blood. However, this did not reach the sensitivity observed in the Darwin study, and we are currently performing PCR on our samples using the Darwin primers and conditions to determine whether the TTS1 assay is more sensitive.

The diagnostic sensitivity for DIF (44%) was lower than that described in a previous evaluation of this test (66%). The study here also highlights the limitations of DIF in that some specimens are not suitable. However, this assay is cheap and quick to perform, and is also a useful assay for the identification of presumptive *B. pseudomallei* growing on solid agar or other culture media.

Comparing costs, PCR, conventional culture, and DIF cost are ~9, 6, and 1.5 USD per sample in our setting, respectively. Real-time PCR requires the initial financial outlay for the PCR machine, together with maintenance costs. Because PCR cannot replace conventional culture in our setting, the debate about whether to introduce this into routine care revolves around affordability, time to positivity, and prognostic value of a positive PCR result. PCR is considerably faster than conventional culture, and our assay had 100% specificity. Furthermore, patients with a positive blood culture who were PCR positive had a 100% mortality rate; such a strong prognostic indicator could be used to identify high-risk patients to whom scarce intensive care resources could be directed. Timing of sampling would be critical; further study is required to determine the effect on outcome of information...
derived from a sample taken for PCR at presentation and prior to antimicrobial therapy.

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