Performance of Nucleic Acid Amplification following Extraction of 5 Milliliters of Whole Blood for Diagnosis of *Mycobacterium tuberculosis* Bacteremia

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To investigate the performance of a nucleic acid amplification test (NAAT) for the diagnosis of *Mycobacterium tuberculosis* bacteremia, 5-ml aliquots of blood were inoculated into bioMérieux mycobacterial (MB) bottles and incubated, and 5-ml aliquots of blood were extracted and tested by real-time PCR. Of 25 samples from patients with *M. tuberculosis* bacteremia, 9 (36.0%) were positive and 1 (1.5%) of 66 control samples was positive by NAAT. The NAAT shows promise, but modifications should focus on improving sensitivity.

Disseminated tuberculosis is a major health problem in countries where generalized HIV epidemics coincide with high tuberculosis incidence rates, often causing fatal illness in patients with immunologically advanced HIV disease (14). Long recognized (5, 13), *Mycobacterium tuberculosis* bacteremia is common in sub-Saharan Africa (1, 2, 4, 16, 20) and Asia (3, 17). In-hospital case fatality rates are high (1), and median survival is short (1, 17, 19). Early recognition and treatment are likely to be important for averting mortality (12). Even when using mycobacterial blood culture systems with continuous detection, the time to positive may be too long to influence clinical management (7, 9). Nucleic acid amplification tests (NAAT) on whole-blood specimens have shown promise for the diagnosis of pulmonary tuberculosis (6, 21). We hypothesized that NAAT on whole blood may be useful for the early diagnosis of the disseminated form of tuberculosis.

Samples for blood cultures, NAAT, and other diagnostic tests were collected from patients aged ≥13 years hospitalized at the Kilimanjaro Christian Medical Centre (KCMC) and Mawenzi Regional Hospital (MRH) in Moshi, Tanzania, from July 2006 through October 2009 (8). Patients with oral temperatures of ≥38.0°C were invited to participate; 5 ml of blood was inoculated into a bioMérieux BacT/Alert MB bottle and 5 ml was inoculated into an EDTA tube for subsequent NAAT. Other study procedures are described elsewhere (7, 8, 10, 15, 23). Only the results of the MB bottle were considered in the classification of cases; a patient with a companion bottle positive for *M. tuberculosis* was not included in the control group even if the MB bottle was negative.

Blood culture bottles and tubes were assessed for volume adequacy by comparing the weights before and after inoculation. A bottle or tube was considered adequately filled if it contained 4 to 6 ml of blood. Only samples from patients with adequately filled bottles and tubes were included in the study. BacT/Alert MB bottles were loaded into the BacT/Alert 3D automated microbial detection system (bioMérieux Inc., Durham, NC) where they were incubated for up to 42 days.

Specimens were classified as being from a case patient with *M. tuberculosis* bacteremia if the MB blood culture bottle was positive for *M. tuberculosis*. Those with mycobacterial blood cultures negative for *M. tuberculosis* were classified as controls. The results of clinical evaluations and examination of nonblood specimens for mycobacteria were available for evaluation following completion of nucleic acid amplification testing (8).

EDTA-blood was transferred to cryovials and stored at −80°C for up to 5 years. Cryovials were shipped on dry ice to the Cleveland Clinic for nucleic acid amplification testing. Each 5-ml sample was thawed, mixed thoroughly, and transferred into an adult Wampole isolator tube (Inverness Medical Innovations, Inc., Princeton, NJ). Each isolator tube was gently vortexed for 5 to 10 s and held at room temperature for at least 1 h to inactivate HIV, if present (11). Following centrifugation at 3,000 × g for 30 min, a pellet was obtained using the manufacturer’s instructions. The 1.5-ml pellet was transferred into a 2-ml Sarstedt microcentrifuge tube and centrifuged at 10,000 × g for 10 min. Approximately 1.2 ml of supernatant was removed, and 500 µl of phosphate-buffered saline (PBS) was added. The suspension was vortexed and centrifuged at 10,000 × g for 10 min, and most of the supernatant was removed. A 180-µl volume of MagNA Pure bacteria lysis buffer (Roche, Indianapolis, IN) and 20 µl of proteinase K (Roche) were added to each pellet, and the mixture was incubated at 65°C for at least 2 h to overnight. The suspension was heated at 100°C for 10 min. Processing of the pellet was performed using a class II biosafety cabinet and a microcentrifuge with a removable rotor. The entire sample was added to 2 ml of NucliSens EasyMAG lysis buffer (bioMérieux, Durham, NC) and extracted on the EasyMAG instrument. A final extraction volume of 50 µl was obtained.

PCR was performed using the LightCycler system (Roche) based on a previously described assay (22), with the following modifications. Asymmetric PCR was used by increasing the re-
verse primer concentration from 0.25 μM to 0.5 μM. Additionally, PCR cycles were increased from 45 to 55, and step mode was selected for melting curve analysis. Positive and negative controls consisted of Mycobacterium ATCC 27294 and PCR-grade water, respectively. If amplification occurred, then the identity of the Mycobacterium species as M. tuberculosis was confirmed using postamplification melt curve analysis by comparison to the positive control (±2°C). Five 1-ml replicates of each sample were tested. All PCR-negative samples were further tested using the LightCycler control kit DNA (Roche), which is a PCR assay for a 110-bp fragment of the human β-globin gene.

Means and ranges were calculated for continuous data and compared by the paired two-sample t test for means after log transformation was performed to correct for the observed positively skewed (nonparametric) distributions. Proportions were compared using the chi-square test with Yates’ correction for small numbers when necessary. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated for the NAAT compared with blood culture. All analyses were done with the SAS system for Windows (release 9.1; SAS Institute, Cary, NC). This study was approved by the KCRC Research Ethics Committee, the Tanzania National Institutes for Medical Research National Research Ethics Coordinating Committee, and an Institutional Review Board of Duke University Medical Center.

Of 91 participants included in the study, 25 (27.5%) had M. tuberculosis bacteremia and were classified as cases. All were HIV infected. The remaining 66 (72.5%) had mycobacterial blood cultures negative for M. tuberculosis and were classified as controls. Characteristics of control participants and samples are summarized in Table 1.

Of 25 samples with M. tuberculosis bacteremia, 9 (36.0%) were positive by NAAT. Of those positive by NAAT, the mean number of replicates that were positive was 3 (range, 1 to 5). For those with results available, the mean magnitude of mycobacteria was 58.1 CFU/ml (range, 17.0 to 90.0) among NAAT-positive samples, compared with 0.5 CFU/ml (mean and range) for NAAT-negative samples (P = 0.157). The mean time to positive in the continuously monitored Bact/Alert MB system was 16.8 days (range, 9.4 to 27.5) for NAAT-positive samples and 22.0 days (range, 11.3 to 30.9) for NAAT-negative samples (P = 0.062) (Table 2).

Of 66 control samples, 1 (1.5%) was positive for M. tuberculosis by NAAT. The sample was positive in 1 of 5 replicates. Evaluation of case report forms showed that this HIV-uninfected participant had clinical features consistent with pulmonary tuberculosis. All 81 PCR-negative samples were β-globin PCR positive, confirming successful specimen DNA extraction and absence of PCR inhibitors.

The sensitivity (95% confidence interval [CI]) of the NAAT for the diagnosis of M. tuberculosis bacteremia was 0.360 (CI, 0.187 to 0.573), and the specificity was 0.985 (CI, 0.907 to 0.999). The positive predictive value (95% CI) of the NAAT for the diagnosis of M. tuberculosis bacteremia was 0.900 (CI, 0.541 to 0.995), and the negative predictive value was 0.802 (CI, 0.696 to 0.879).

We found that extraction of 5 ml of whole blood followed by real-time PCR targeting of the mycobacterial 16S rRNA gene (22) detected approximately one-third of patients with M. tuberculosis bacteremia diagnosed by culture of an equivalent volume of blood. Specificity exceeded 98% in a control population that included HIV-infected persons enrolled in a country with a high incidence of tuberculosis. There was a trend toward patients with a higher magnitude of mycobacteremia being more likely to have a positive M. tuberculosis NAAT result.

Our NAAT was less sensitive in patients with confirmed M. tuberculosis bacteremia than an IS6110-based assay with patients with suspected pulmonary tuberculosis (6, 21). Possible explanations include that extracting whole blood rather than buffy coat may have increased the effect of blood-associated PCR inhibitors; this may have been compounded by the use of frozen rather than fresh whole blood. Differences in sensitivity may also relate to the nested design of the IS6110-based assay (6, 21) combined with a higher copy number of the IS6110 target compared with the mycobacterial 16S rRNA gene (22). Our assay may perform better in patients with higher magnitudes of mycobacteremia (7).

Despite studying a population with a high seroprevalence of HIV (8) and risk for pulmonary tuberculosis (18), the specificity of our assay was relatively high (6, 21). Efforts to increase the sensitivity of our NAAT may result in the detection of more patients with pulmonary tuberculosis and low-magnitude M. tuberculosis bacteremia not detected by blood culture. Specimens from control patients with a range of bacterial and fungal bloodstream infections were negative in the M. tuberculosis NAAT, confirming the specificity of the assay in the presence of a range of epidemiologically important conditions (20, 22).

In conclusion, we have demonstrated that a NAAT approach could provide a solution to the rapid diagnosis of bacteremic disseminated tuberculosis. Although our assay lacked sensitivity, the potential to detect more than a third of patients with M. tuberculosis bacteremia represents an important step forward in laboratory diagnosis of a condition that is rapidly fatal in a large propor-

**TABLE 1 Patient samples with and without M. tuberculosis bacteremia selected for evaluation of the nucleic acid amplification test**

<table>
<thead>
<tr>
<th>Designation</th>
<th>HIV serostatus</th>
<th>Invasive infection category</th>
<th>Bloodstream isolate</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case</td>
<td>Infected</td>
<td>Met study eligibility, M. tuberculosis bloodstream infection</td>
<td>M. tuberculosis</td>
<td>25 (27.5)</td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>Nontuberculous mycobacterial bloodstream infection</td>
<td>Mycobacterium sherrisii (1), Mycobacterium simiae (1)</td>
<td>2 (2.2)</td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>Met study eligibility, blood culture negative</td>
<td>Negative</td>
<td>13 (14.3)</td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>Nontuberculous mycobacterial bloodstream infection</td>
<td>Cryptococcus neoflammans (5), Escherichia coli (3), Strepococcus pneumoniae (1)</td>
<td>9 (9.9)</td>
</tr>
<tr>
<td></td>
<td>Uninfected</td>
<td>Nontuberculous mycobacterial bloodstream infection</td>
<td>E. coli (4), S. pneumoniae (2), Salmonella enterica serovar Typhi (16)</td>
<td>22 (24.2)</td>
</tr>
<tr>
<td></td>
<td>Uninfected</td>
<td>Blood culture negative</td>
<td>Negative</td>
<td>20 (22.0)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>91 (100)</td>
</tr>
</tbody>
</table>

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We recommend that future work be focused on improving the lower limit of detection of the assay.

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REFERENCES

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