Direct Detection of Mycobacterium sp in Respiratory Specimen with rpoB-PCR and Comparison with Concentration Fluorochrome Staining

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Abstract: The purpose of the present study was to evaluate the usefulness of rpoB-PCR assay and concentration flurochrome (CF) staining against culture for direct detection of mycobacteria sp in respiratory specimen among symptomatic patients. Of the 900 specimens 52 were culture positive with Löwenstein-Jensen Medium (LJM). Of the 52 culture positive samples, 48 were positive by rpoB-PCR (sensitivity 92.3%) and 46 of culture positive specimens were positive by CF staining (sensitivity 88.4%). On the other hand, of the 848 culture negative samples, 5 were positive by rpoB-PCR (specificity 99.5%) and 3 were positive by CF staining (specificity 99.6%). Both of tests are highly sensitive and specific for direct detection of mycobacteria sp from respiratory specimen within hours and CF staining should be recommended for routine use in the clinical microbiology laboratory in developing countries, where limited resources for usage of molecular techniques for rapid detection of tuberculosis.

Key words: rpoB-PCR, direct detection, tuberculosis, respiratory specimen, culture

INTRODUCTION

Tuberculosis (TB), a chronic infection, remains the second most common infectious disease worldwide with the 8-12 million new cases and nearly 2 million deaths occurring each year[1,2]. It was estimated that 2 billion individuals already infected with Mycobacterium tuberculosis and 4.6 million of the world population are coinfected with human immunodeficiency virus (HIV) and M. tuberculosis[1]. Advances in rapid diagnostic methods and direct differentiation of mycobacteria sp, especially in immunocompromised patients, are urgently needed for the early management and successful treatment patients and TB control[3-4]. However the definitive diagnosis of tuberculosis depend on isolation of mycobacteria sp from patient’s specimen but access to culture’s results may require several weeks[2]. Usefulness of PCR assay for rapid detection of tuberculosis have reported by a number of investigators but its ability for direct detection of mycobacteria sp depend on, operational skill, PCR methods, target region and detection system[5]. However two of nucleic acid amplification tests methods, Mycobacterium Tuberculosis Direct Test (MTD; Gen-Probe, San Diego, Calif.) and Amplicor (Roche Diagnostic Systems, Somerville, N.J.) were approved in 1996 by the Food and Drug Administration for direct detection of Mycobacterium tuberculosis complex in respiratory specimens, but they can not detect nontuberculosis mycobacterium (NTM) in clinical specimens[6]. In this study explores the potential utility of rpoB-PCR assay for rapid direct detection of mycobacteria sp in respiratory specimen and comparison with concentration flurochrome staining.

MATERIALS AND METHODS

From 2002 to 2005, 900 respiratory samples of patients were admitted to microbiology laboratory of Tehran University of medical sciences. Respiratory specimens were first liquefied and decontaminated with 0.5% N-acetyl-L-cysteine, 2% NaOH, and 1.45% Sodium Citrate. Following vortexing, the specimens were concentrated by centrifugation at 3,000 × g for 15 minute[7]. Then the sediments were resuspended in sterile normal saline solution, and used for concentration flurochrome (CF) staining, culture, and PCR studies.

Concentration Fluorochrome (CF) staining: Three drops of sediment (approximately 0.15 ml) were placed on a slide and heat fixed on a slide warmer at 75°C for approximately 2 h. The slide was stained with auramine-rhodamine stain and examined with an optical microscope with a 25× objective under a standard fluorescence UV filter. High power (100× objective) microscopy was used when confirmation of morphology was needed. Each slide was examined for the presence of fluorescent bacilli by making passes along the long axis of the slide. At least one bacillus in any examination was rated as positive[7].

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Löwenstein-Jensen culture: The processed specimens were inoculated on Löwenstein-Jensen medium (0.1 to 0.2 ml of sediment) and incubated for 8 weeks. Because of proven high sensitivity of culture, this technique was used as the gold standard in this study. All samples that gave a positive culture were checked by the CF staining, to confirm the growth of Acid Fast Bacilli (AFBs). Negative culture was discarded after 8 weeks.

DNA extraction: DNA extraction was performed by SDS/Lysozyme method. Two hundred and fifty microlitres of concentrated specimens were resuspended in a final volume of 1 ml of Tris-EDTA, pH 7.6 containing 10 mg of lysozyme and incubated at 37°C for 1 h, 30 µL of solution that contain proteinase K (14 mg/ml) and SDS (3%) were added, followed by incubation for 2 to 3 h at 56°C or overnight at 37°C. Proteinase K was inactivated by 15 min of incubation at 95°C. To monitor for cross-contamination, one water-containing negative control tube was used[8].

RpoB-PCR: A set of mycobacteria-specific primers (MF, 5’CGACCACCTCGGCACCCG3’; MR, 5’TCGATCCGGGCACATCCGG3’) was used to amplify rpoB DNA (342 bp). The primers were selected from the highly conserved regions. Template DNA and 20 pmol of each primer were added to a 0.5 ml PCR mixture tube which contained 1U of Taq DNA polymerase, 250 µM each dNTP, 50 mM Tris-HCl (pH 8.3), 1.5 mM MgCl and the volume was adjusted to 25µl with distilled water. The reaction mixture was subjected 30 cycles of amplification (30s at 95°C, 30s at 60°C and 45s at 72°C) followed by a 5 min extension at 72°C. The amplicons were detected by gel electrophoresis using 1.5% agarose gel with ethidium bromide[9]. To avoid cross contamination and false positive in the rpoB-PCR assay, we used three separate areas: a reagent preparation, a specimen preparation and an amplification and detection room[9].

RESULTS AND DISCUSSIONS

Nucleic acid amplification (NAA) tests and CF staining allow the detection of mycobacteria in specimens within hours, compared with the 2-4 weeks usually required for culture[10]. Since no reliable rapid resistant detection system is now available for mycobacteria, culture methods are still needed for routine diagnosis and susceptibility testing and NAA does not replace the need to perform cultures[11]. Our study attempted to evaluate the applicability of the rpoB-PCR assay and FC staining tests in countries where tuberculosis is highly prevalent and atypical mycobacteria are less seen. We compared the performance of the rpoB-PCR assay with that of the CF staining for the direct detection of mycobacteria sp in respiratory specimens. From 52 (5.7%) of the 900 specimens, mycobacteria were cultured. Of 52 culture positive specimens 48 were rpoB-PCR positive (sensitivity 92.3%) and 46 of them were positive by CF staining (sensitivity 88.4%). 46 of 49 smears that were found positive by the CF staining were confirmed with rpoB-PCR assay and culture method. Of 6 culture positive specimens that were considered negative with the CF staining, 2 were positive in rpoB-PCR assay. 3 cultures and rpoB-PCR negative patients’ specimens were positive by CF staining (specificity 99.6%) and 5 patients positive by rpoB-PCR assay failed to grow mycobacteria on LJ medium (specificity 99.5%). Calculated CF stained smears positive predictive value (PPV) and negative predictive value (NPV) were 93.8 and 99.2 %, respectively. As for rpoB-PCR assay, the calculated PPV and NPV were 90.5 and 99.5 %, respectively. The results obtained from culture, CF stained smears and rpoB-PCR methods were summarized in the Table 1. NAA, but not CF staining, on clinical specimens may also be useful when there is a requirement for rapid differentiation between mycobacteria species. Utility of this system to differential identification of mycobacterial sp based on PCR

Table 1: Sensitivity, specificity, PPV and NPV of rpoB-PCR and concentration microscopy techniques

<table>
<thead>
<tr>
<th></th>
<th>No. of cultures with mycobacteria</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (n=900)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rpoB-PCR</td>
<td>Positive</td>
<td>92.3</td>
<td>99.5</td>
<td>90.5</td>
<td>99.5</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>4</td>
<td>843</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>48</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
<td>843</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration microscopy</td>
<td>Positive</td>
<td>88.4</td>
<td>99.6</td>
<td>93.8</td>
<td>99.2</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>46</td>
<td>3</td>
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<td></td>
</tr>
<tr>
<td>Positive</td>
<td>6</td>
<td>845</td>
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restriction-fragment length polymorphism (PCR-RFLP) is the main advantage over other currently procedure available molecular tests for direct detection of mycobacteria[13]. Using different target sequences, extraction and detection methods have produced very variable outcomes in direct detection of mycobacteria, with diagnostic sensitivity ranging from 65.2 to 100%[4,5,12-19]. However, Study of Ichiyama et al.[5]. Revealed that AMTD, an RNA amplification-based Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test system had 100% sensivity to direct detection of mycobacteria but it can detect only Mycobacterium tuberculosis.

We and other investigators presented the sensitivity of the direct NAA as equal as or greater than that in the culture[4,13,20-23]. In our study, we have shown that 5 (9.6%) false positive in rpoB-PCR assay. False-positive results by the NAA have also been reported for specimens collected from patients who were undergoing antituberculosis treatment. However, the decontamination methods can kill mycobacteria and thus produce false positive PCR results. The results of NAA, same as the other laboratory findings, should always be interpreted according to clinical data[24]. After the clinical resolution of PCR false-positive caseS, the specificities of these systems will be increased[5]. False-negative results of the rpoB-PCR assay (7.6%) and CF staining (11.5%) were considered to be due to the low number of mycobacteria and/or the presence of inhibitors in the specimen[5,23,24]. The data reported here show the outstanding sensitivities (92.3%) and specificities (99.5%) of the rpoB-PCR assay. This system is also easy to perform and making its ideal tool for rapid direct detection of mycobacteria in respiratory specimens. Our current AFB smear positivity rate for mycobacteria detection (88.4%) was higher than the other studies (62.5-66%)[6,25] but the specificity of this method was as the same as another study[26]. Surprisingly, in this study sensitivity of CF staining in direct detection of mycobacteria was not very different than rpoB-PCR. The sensitivity and specificity of this test has improved considerably with improved techniques, standardization of sputum preparation and experience microbiologists[27]. According to the present study concentration fluorochrome microscopy remains the most available, easy to perform, inexpensive, rapid, sensitive and specific diagnostic test for pulmonary tuberculosis. This is especially true for laboratories in developing countries, where limited resources for usage of molecular techniques for rapid detection of tuberculosis.

REFERENCES


