Short Communication

Direct Early Identification of *Mycobacterium tuberculosis* by PCR-Restriction Fragment Length Polymorphism Analysis from Clinical Samples

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**SUMMARY:** We attempted to apply the PCR-restriction fragment length polymorphism (PCR-RFLP) technique for the early detection and identification of *Mycobacterium tuberculosis* directly from clinical samples. PCR-RFLP of *hsp65* was applied to the DNA extracted directly from sputum samples (*n* = 226) collected from 226 patients. We could detect and identify *M. tuberculosis* in 84.5% of the acid-fast bacillus (AFB) smear-positive samples (*n* = 149) and 11% of the AFB smear-negative samples (*n* = 18) obtained from patients with clinical and radiological evidence of tuberculosis. Sputum samples (*n* = 59) obtained from patients suffering from respiratory diseases other than tuberculosis were included as negative controls. To test the sensitivity of the assay, we spiked a smear-negative sample with serial dilutions of H37Rv. The protocol could detect down to 10 organisms/μl. PCR-RFLP was found to be a simple and reproducible method for early detection of *M. tuberculosis* from sputum samples. The present assay could be used to augment conventional methods of diagnosis of mycobacterial diseases and thus help clinicians to differentiate between *M. tuberculosis* complex and non-tuberculous mycobacteria directly in clinical samples. The assay could help clinicians to select appropriate chemotherapeutic agents early, which could considerably reduce the morbidity due to mycobacterial diseases.

Early mycobacterial identification to the species level is important because it would help in the initiation of early and appropriate treatment of patients. However, identification of mycobacteria by conventional methods is time-consuming and not always conclusive (1,2). Other methods such as high-performance gas-liquid and thin-layer chromatographies and DNA sequence analysis of the 16S rRNA gene (rDNA) region are labor-intensive, difficult, and expensive for routine use (3–5). Rapid and simple genotypic assays for the identification of mycobacteria, such as Accuprobe (Gen-Probe Inc., San Diego, Calif., USA), are available commercially, but the high costs of these assays have restricted their large-scale use in most clinical laboratories, especially in high-burden countries endemic for tuberculosis (6). Newer techniques such as loop-mediated isothermal amplification (LAMP) and PCR-restriction fragment length polymorphism analysis (PCR-RFLP) are simple to perform, easy to read, reproducible, and rapid, which are features that make them highly attractive for use in routine clinical laboratories (6,7). PCR-RFLP, in addition, can differentiate numerous species of mycobacterium within a single experiment.

PCR-RFLP techniques have been developed for several mycobacterial genes, such as *hsp65*, the 16S-23S rDNA spacer, *dnaJ*, and *rpoB* (6.8). Of these, the one most investigated and validated is *hsp65* (6,9). However, *hsp65* gene-based PCR-RFLP has been impeded by difficulties such as minor differences in band sizes between some species and the occurrence of new patterns that have not been reported previously (6). Wong et al. (6) designed an assay for PCR-RFLP of the *hsp65* gene using the restriction enzymes *CfoI* and *Sau96I* so that *Mycobacterium tuberculosis* complex, *Mycobacterium avium* complex (MAC) species, and other clinically important mycobacteria can be identified from positive cultures. The restriction pattern of *M. tuberculosis* was highly distinct by this method, and the patterns could be identified visually (6). Moreover, the reliability of PCR-RFLP results with one enzyme is augmented if the results are confirmed with a second restriction enzyme (10). In the present study, we applied the PCR-RFLP method of Wong et al. (6) directly on sputum samples rather than on culture-positive *M. tuberculosis* isolates, as was reported by them. The long-term aim was to use PCR-RFLP to identify and differentiate *M. tuberculosis* from non-tuberculous mycobacteria (NTM) directly in clinical samples using a technique that would not only be rapid but also simple enough to be used in a non-referral laboratory so it could thus be used to augment conventional techniques in a mycobacteria diagnostic laboratory.

Two hundred and twenty-six sputum samples were collected between September 2005 and December 2007. Of these, 167 samples were obtained from clinically suspected patients of pulmonary tuberculosis admitted at Rajan Babu TB Hospital, Delhi, India. Rajan Babu TB Hospital serves as a referral center for patients of tuberculosis in North India. Fifty-nine sputum samples were collected from patients suffering from chronic bronchitis, acute exacerbations of chronic obstructive pulmonary disease (COPD), and bronchial asthma, but not tuberculosis, attending the Vallabhbhai Patel Chest Institute (VPCI), Delhi, India. VPCI is a referral hospital in North Delhi for chest diseases.

Of the 167 sputum samples from patients of pulmonary tuberculosis, 149 were acid-fast bacillus (AFB) smear-positive. The smears were graded and the samples cultured on Lowenstein-Jensen medium according to the protocols recommended by the Revised National Tuberculosis Control Programme (RNTCP), India (11) (Table 1). Positive-culture isolates were subjected to biochemical reactions by the standard procedure (1).
The sputum samples were prepared for PCR by mixing equal amounts of the sample and 4% NaOH and incubating the mixture for 10 min at 37°C. The mixture was then centrifuged, and the pellet was washed thrice with PBS (pH 7.4). The pellet was finally suspended in 1% Triton X-100, and the suspension was boiled for 30 min. The lysate was centrifuged, and the supernatant was collected for PCR. DNA was extracted from the reference strain H37Rv and 60 randomly selected positive cultures by the cetyltrimethylammonium bromide (CTAB) method, as described previously (12).

PCR restriction analysis was performed according to Wong’s protocol (6). A 294-bp amplicon with the hsp65 primers was obtained in 126 (84.5%) of the 149 AFB smear-positive samples (Table 1). Of the 18 AFB smear-negative samples obtained from patients with suspected tuberculosis, 2 (11%) were PCR positive. No PCR amplicon was obtained in any of the 59 sputum samples obtained from patients suffering from respiratory diseases other than tuberculosis. All the samples showing a positive amplification product were further subjected to restriction digestion with Sau96I and CfoI. Analysis of the restriction patterns showed a banding pattern identical to H37Rv in all the clinical samples. Sau96I restriction produced 2 bands at 219–240 bp and 54–75 bp. Digestion with CfoI yielded 3 bands at 122, 83, and 72 bp (Fig. 1A). Finally, the presence of M. tuberculosis was detected by PCR-RFLP in 76.6% sputum samples obtained from patients of tuberculosis (128/167) (Table 1). Neither culture nor PCR-RFLP in 76.6% sputum samples obtained from patients suffering from respiratory diseases other than tuberculosis. All the samples showing a positive amplification product were further subjected to restriction digestion with Sau96I and CfoI. Analysis of the restriction patterns showed a banding pattern identical to H37Rv in all the clinical samples. Sau96I restriction produced 2 bands at 219–240 bp and 54–75 bp. Digestion with CfoI yielded 3 bands at 122, 83, and 72 bp (Fig. 1A). Finally, the presence of M. tuberculosis was detected by PCR-RFLP in 76.6% sputum samples obtained from patients of tuberculosis (128/167) (Table 1). Neither culture nor PCR-RFLP detected any NTM in the samples taken for this study. Sixty sputum samples were also tested for the presence of the human β-actin gene, which served as an internal control (13).

To test the sensitivity of the assay for identifying M. tuberculosis, we spiked a smear-negative sputum sample with serial dilutions of H37Rv in a spiked smear-negative sputum sample. Lane 1 is a 50-bp DNA marker. Lanes 2 and 5 show the undigested PCR-products from DNA extracted from the laboratory strain H37Rv and the clinical specimen, respectively. Lanes 3 and 6 represent the Sau96I digests from H37Rv and the clinical specimen, respectively. Lanes 4 and 7 represent the CfoI digests from H37Rv and the clinical specimen, respectively. (B) PCR products obtained from serial dilutions of H37Rv in a spiked smear-negative sputum sample. Lane 1 is a 50-bp DNA marker. Lanes 2-8 show the PCR amplicons from samples with 10³, 10⁴, 10⁵, 10⁶, 10⁷, 10⁸, and 10⁹ organisms/μl, respectively.

Table 1. Summary of PCR-RFLP results in relation to acid-fast bacillus (AFB)-smear grading of sputum samples obtained from patients of tuberculosis

<table>
<thead>
<tr>
<th>AFB-smear grading</th>
<th>No. of samples</th>
<th>No. of samples identified as Mycobacterium sp. on direct PCR-RFLP (%)</th>
<th>No. of cultures demonstrating M. tuberculosis with PCR-RFLP</th>
<th>No. of sputum samples demonstrating M. tuberculosis with direct PCR-RFLP/No. demonstrating M. tuberculosis in culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1+</td>
<td>50</td>
<td>38 (76)</td>
<td>25/25</td>
<td>23/25</td>
</tr>
<tr>
<td>2+</td>
<td>45</td>
<td>39 (86.6)</td>
<td>19/19</td>
<td>16/19</td>
</tr>
<tr>
<td>3+</td>
<td>54</td>
<td>49 (90.7)</td>
<td>16/16</td>
<td>13/16</td>
</tr>
<tr>
<td>Negative</td>
<td>18</td>
<td>2 (11)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>167</td>
<td>128 (76.6)</td>
<td>60/60</td>
<td>52/60</td>
</tr>
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</table>

1). Direct PCR-RFLP had been performed on the corresponding sputum samples on the day of receipt of the samples, 30 days before the culture was reported positive.
chemical reactions and showed an RFLP pattern of *M. tuberculosis*. Our results highlight the efficacy of the present method by showing that the direct PCR-RFLP from corresponding sputum samples revealed the presence of *M. tuberculosis* in 52 of the samples, 30 days before the culture report was available.

Though PCR-RFLP in our study had a lower sensitivity (76.6%, 128/167), as compared to Ziehl-Neelsen staining (89%, 149/167), PCR-RFLP scored over conventional techniques in the rapid detection and speciation of *M. tuberculosis* directly from the samples. The advantage of direct PCR-RFLP was that it needed just 24 h for identification after sample collection, whereas biochemical reactions required at least 4 weeks from the time of first inoculation to speciation for final identification. Moreover, though several tuberculosis-specific PCR assays have been validated using the IS6110 gene actin gene as the target, the advantage of the present protocol lies in the fact that it can be used in regions with a low (<6) number of IS6110 bands or no IS6110 bands, as have been reported from India (16,17). In addition, unlike certain new technologies such as LAMP, which require different primer sets to identify various NTM, PCR-RFLP can identify a vast range of mycobacterial species in a single experiment. In addition, new species with different restriction patterns would easily be identified by PCR-RFLP. The potential reasons why PCR-RFLP could not detect *M. tuberculosis* in a significant proportion of smear-positive samples (15.4%, 23/149) could be faulty extraction or PCR inhibition (Table 1). We tested 60 sputum samples for PCR inhibitors by using PCR for the β-actin gene as an internal control. No PCR product was found in 20% of samples, thus implying that these samples probably had PCR inhibitors.

To conclude, PCR-RFLP is highly specific, and the cost of an assay is as low as $1.50 per test. Administration of the assay does not require any high-cost instrumentation or technical expertise. PCR-RFLP could, thus, prove to be a suitable rapid method for identification of *M. tuberculosis* directly from clinical samples.

**ACKNOWLEDGMENTS**

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**REFERENCES**