PCR amplification of shorter fragments from the devR(Rv3133c) gene significantly increases the sensitivity of tuberculosis diagnosis

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Abstract

This study was designed to assess the vital issue of gene target length and PCR assay performance in relation to the detection of Mycobacterium tuberculosis in clinical specimens. Two PCR assays that amplify fragments of varying lengths from the devR gene of M. tuberculosis were evaluated. Using M. tuberculosis DNA the ‘short-length’ PCR assay detected 250–500 genome equivalents vs. 500–1000 genome equivalents by the ‘long-length’ assay. In comparison to a highly sensitive smear microscopy test (universal sample processing smear), the sensitivity of the ‘short-length’ assay was 97.8% vs. 69.9% of the ‘long-length’ assay in sputum specimens (n = 506) from patients being evaluated for a possible diagnosis of tuberculosis. The 27.9% absolute increase in sensitivity was statistically significant (P < 0.001).

Our results indicate that in a clinical setting when all other conditions are equal, the amplification of a shorter gene fragment of devR increases the sensitivity and efficiency of the PCR assay in spite of using a single copy gene as target.

Introduction

Nucleic acid-based technologies such as PCR offer great promise for the rapid, sensitive and specific diagnosis of infectious diseases including tuberculosis in situations where conventional approaches fail because of prolonged incubation times for culture and lack of sensitivity of smear examination. Numerous gene targets for PCR-based diagnosis of tuberculosis have been developed and are under continuous evaluation (Tumwasorn et al., 1996; Marchetti et al., 1998; Singh et al., 1999; Saves et al., 2002). In our laboratory, we developed a Mycobacterium tuberculosis complex-specific PCR assay based on the devR gene wherein a 513 bp long fragment was amplified using devRf/devRr primers (Singh et al., 1999, 2000). This gene encodes a response regulator that was identified earlier as being differentially expressed in the virulent strain of M. tubercu-

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Materials and methods

Patients

This study was performed on 506 sputa collected from 506 patients visiting the Microbiology and DOTS Center at the New Delhi Tuberculosis Center, New Delhi; Sunderlal Jain Charitable Trust Hospital, Delhi; and Tuberculosis Research Center, Chennai (TRC), for diagnosis of pulmonary tuberculosis. The details on patient selection were provided earlier (Chakravorty et al., 2005). Briefly, the patients were evaluated for any of the following clinical symptoms: fever, cough, expectoration of sputum, haemoptysis, pain, dyspnoea, loss of weight, night sweats, general weakness, X-ray chest, Mantoux status and any past history of tuberculosis. Any specimen collected from a subject already on antitubercular therapy at the time of specimen collection was excluded from the study.

USP methodology

Sputum samples were processed by USP methodology as described earlier (Chakravorty & Tyagi, 2005; Chakravorty et al., 2005). Briefly, sputum samples were homogenized with 1.5 to two volumes of USP solution (4–6 M guanidinium hydrochloride, 50 mM Tris-Cl, pH 7.5, 25 mM ethylenediamine tetra-acetic acid, 0.5% Sarcosyl, 0.1–0.2 M β-mercaptoethanol) for 5–10 min at room temperature, after which 10–15 mL of sterile water or 68 mM phosphate buffer pH 6.8 was added and mixed. The sample was centrifuged at 5000–6000 g at room temperature for 10–15 min and the sediment was washed once more with USP solution followed by a wash with 10 mL sterile triple-distilled water. The sediment was resuspended in 500 μL of resuspension solution and used for smear microscopy (10%), culture on Lowenstein–Jensen slopes (50%) and DNA isolation (40%) as described. All the slides were subjected to Ziehl–Neelsen (ZN) staining. Cultures were confirmed to be Mycobacterium tuberculosis by the niacin test or by devR PCR as described (Singh et al., 2000).

DNA isolation and PCR

A unidirectional workflow scheme and physical separation of DNA isolation, amplification and analysis sites was adopted to minimize false-positive results from PCR assays. DNA was isolated from USP-processed sediments by boiling them in the presence of five times the pellet volume of 10% Chelex 100 resin, 0.03% Triton X-100 and 0.3% Tween-20 as described (Chakravorty & Tyagi, 2005). An aliquot was used in the ‘long-length’ PCR assay as described (Singh et al., 1999, 2000) and in a ‘short-length’ assay that amplified a 308 bp region of the devR gene using devRF2 (5′-TGGCAACGGCATTTGAACTGT-3′) and devRr2 (5′-TAAGCAGGCCAGTAGCGT-3′) primers. Briefly, DNA was amplified in a reaction mixture containing 1 × PCR buffer, 1.5–2.0 mM MgCl₂, 0.5 μM of each primer, 0.2 mM dNTPs, 1 U of Taq polymerase and 4 μL (genomic DNA) or 10 μL (clinical specimen DNA) of template per 40 μL PCR reaction. The thermal cycling parameters were 10 min at 94 °C, 45 cycles each of 1 min at 94 °C, 1/1.5 min at 52/65 °C (for ‘short-length’/‘long-length’ assays), 30 s at 72 °C for the ‘short length’ assay only and a final extension of 7 min at 72 °C for both the assays. The amplified products were visualized by ethidium bromide staining after agarose gel electrophoresis. An additional PCR assay targeting the repetitive IS6110 sequence was also carried out on the DNA isolated from all the samples as described before (Eisenach et al., 1990) to see how the single-copy devR assay performs with respect to this multicopy target.

Statistical analysis

Test results were classified as true positives (TP), true negatives (TN), false positives (FP) and false negatives (FN). The sensitivity and specificity of PCR were calculated using either culture or USP smear positivity as the gold standard. Sensitivity was calculated as TP/TP + FN × 100 and specificity as TN/TN + FP × 100. Efficiency (Armitage, 1971) was calculated as [TP + TN obtained/Total sample number] × 100 as described. McNemar’s test (Rosner, 2000) was performed to determine the statistical significance of the difference observed between the PCR assays.

Results

Performance of long length vs. short length PCR assays using Mycobacterium tuberculosis DNA

The long length assay was first standardized with respect to the annealing temperature (65 and 52 °C) and cycle steps (2-step vs. 3-step) of the PCR reaction. The sensitivity and specificity parameters of the 2-step PCR at an annealing temperature of 65 °C were superior to that of the 3-step PCR performed at the same temperature and also to that of the 3-step PCR carried out at an annealing temperature of 52 °C (data not shown). The standardized assays were used in the present study (details are provided in Materials and methods). The detection limit and specificity of the assays were first determined on serial dilutions of purified Mycobacterium tuberculosis H37Rv DNA. The ‘short-length’ assay was ~2–4 fold more sensitive than the ‘long-length’ assay and could reproducibly detect 250–500 genome equivalents by ethidium bromide staining after agarose gel electrophoresis (Table 1). Samples containing 250 genome equivalents were found to be positive six out of the eight times tested. Similar to the ‘long-length’ assay, the ‘short-length’ assay also showed excellent specificity; the specific 308 bp
amplification product was obtained only with DNA from *M. tuberculosis* complex organisms (Fig. 1). The PCR assay based on the multicopy IS6110 target (16 copies in the H37Rv genome) showed a better sensitivity than both the ‘short’ and ‘long’ length assays with the panels of purified *M. tuberculosis* DNA (Table 1), as expected.

**Table 1.** Comparative sensitivity of devR ‘short-length’, ‘long-length’ and IS6110 assays using serial dilutions of Mycobacterium tuberculosis DNA.

<table>
<thead>
<tr>
<th>Input DNA in PCR assay (c. genome equivalents)</th>
<th>300 ng</th>
<th>50 ng</th>
<th>10 ng</th>
<th>1 ng</th>
<th>100 pg</th>
<th>10 pg</th>
<th>5 pg</th>
<th>2.5 pg</th>
<th>1 pg</th>
<th>100 fg</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Long-length’ (513 bp)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+?*</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>‘Short-length’ (308 bp)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+?*</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IS6110 (123 bp)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Not consistently amplified (6–7/8 times tested).
Primers described by Eisenach et al. (1990) were used.

**Performance of ‘long-length’ and ‘short-length’ PCR assays in a clinical setting**

These assays were evaluated next on 506 sputum specimens as described in Materials and methods. PCR inhibition was ruled out through inhibitor-check reactions containing *M. tuberculosis* DNA. Considering culture as the Gold standard, the ‘long-length’ assay showed a sensitivity of only 74%; in fact 71 culture-positive samples were missed by this assay (Table 2). In contrast, the ‘short-length’ assay showed excellent sensitivity at 98.5% and only four culture-positive samples were missed by it (Table 2). By reducing the amplicon length, the devR ‘short-length’ assay’s sensitivity matched that of the multicopy IS6110 assay (99.6% sensitivity, Table 2). Thus, the application of the ‘short-length’ PCR increased assay sensitivity by 24.5% (absolute percentage increase), and this difference was significant ($P < 0.001$). However, the specificity of the ‘short-length’ assay was only 77.7% compared with 91.9% of the ‘long-length’ assay (Table 2).

In this study, smear-positive specimens outnumbered the culture-positive samples (312 vs. 273, respectively, Table 2); thus 39 samples were smear positive but culture negative and none of the culture-positive samples went undetected by the USP smear microscopy. The detection of additional specimens by the smear test is attributed to the use of highly sensitive USP smear microscopy, whose lower limit of detection is ~300 bacilli per mL of sputum. The superiority of USP smear microscopy over culture was amply demonstrated on sputum in a clinical setting (Chakravorty & Tyagi, 2005; Chakravorty et al., 2005). We do not believe that the discrepancy between smear and culture results was due to the inclusion of some patients on undisclosed prior therapy as every effort was made to exclude this category of patients. However, this possibility cannot be formally ruled out. The inferior performance of culture relative to USP smear highlighted the limitation of using culture as the gold standard for assessing the performance of PCR. Furthermore, the use of culture as the gold standard is handicapped by the time delay of 6–8 weeks to obtain results on conventional solid culture media. National tuberculosis control programmes worldwide, including the Revised National Tuberculosis Control Programme in India (RNTCP), use smear positivity to diagnose active tuberculosis as the yield of nontuberculous mycobacteria from symptomatic subjects in such settings is negligible. In fact, when acid-fast bacilli smear and direct amplification tests are both positive, the diagnosis of tuberculosis is considered as established in culture-negative samples (American Thoracic Society, 1997). Therefore, we compared the performance of PCR with that of USP smear microscopy. Now the specificity of the ‘short-length’
devR assay improved from 77.7% to an acceptable 91.8% (Table 2). The specificity of the ‘long-length’ assay also increased marginally from 91.9% to 96.9%.

Analysis of the culture-positive and USP smear-positive specimens that were negative by the ‘long-length’ PCR assay revealed that they were of ‘scanty’ direct smear grade status. Thus, the ‘long-length’ assay did not efficiently detect paucibacillary specimens and this deficiency was remedied using the ‘short-length’ assay. We also observed that the IS6110 and devR ‘short-length’ assays performed equally well inspite of the former being a multicopy target vs. the latter target, which is present in a single copy in the M. tuberculosis genome (sensitivity was 99.6% vs. 98.5%, respectively, Table 2).

**Discussion**

Published studies that compared the performance of various assays targeting different genes in the same clinical specimen showed that assays that targeted single copy genes and amplified longer fragments were less sensitive than those directed towards repetitive sequences or shorter targets (Marchetti et al., 1998; Grosser et al., 1999; Saves et al., 2002), particularly in paucibacillary specimens and samples containing degraded DNA. In the present study, the IS6110 assay (which amplified a 123 bp fragment) was at least 50-fold more sensitive than the ‘short-length’ devR assay when purified DNA was used (Table 1). Yet the IS6110 and devR ‘short-length’ assays performed equally well on sputum. However, caution is to be exercised while using repetitive sequences like IS6110 as the sole assay target in view of reports that this element was either missing or present as a single copy in a significant proportion of clinical isolates from India (Radhakrishnan et al., 2001; Narayanan et al., 2002). On the other hand, as devR was present in all Mycobacterium tuberculosis-containing specimens and clinical isolates examined so far in our laboratory, it would be a superior gene target over IS6110 (unpublished observations). Our study demonstrates that the disadvantage of devR over IS6110 in terms of copy number was overcome successfully by amplifying a shorter segment of DNA, and this is a major advantage of the ‘short-length’ assay.

The only difference between the two devR assays was the product length generated during amplification. Therefore, we attribute the relatively poor performance of the ‘long-length’ PCR to the possible occurrence of DNA fragmentation, which leads to a decrease in the yield of intact DNA. Fragmented DNA is a relatively poor template for efficient amplification of longer DNA products compared with shorter segments of DNA, and it could occur during or even before DNA isolation as reported in archival paraffin-embedded specimens (Marchetti et al., 1998; Grosser et al., 1999). Furthermore, the faster reaction kinetics during amplification of the shorter fragment compared with the longer fragment is a possible reason for the preferentially better sensitivity of the former assay.

A matter of concern was the status of samples that were detected as PCR positive among USP smear-negative samples. There were 6, 16 and 27 such samples in the ‘long-length’, ‘short-length’ and IS6110 assays, respectively (Table 2). At first glance these can be considered to be false positives. False positives can be attributed to technical factors like possible cross contamination at the sample level as they were collected from a routine microbiological laboratory handling a large number of specimens or carry over contamination during PCR as the uracil N-glycosylase-dUTP system was not used. Furthermore, uneven distribution of the tubercle bacilli because of their inherent clumping nature could have occurred in spite of the precautions taken during processing, among the aliquots processed for smear microscopy, culture and PCR, and may have resulted in an insufficient number of bacilli suitable only for detection by PCR and not by smear microscopy or by culture. From a clinical standpoint, in a disease-endemic country like India, a significant number of persons are infected with M. tuberculosis but without any clinical signs. It has been reported that culture negative specimens, obtained from previously undetected tuberculosis patients, were positive by PCR (Beige et al., 1995). Furthermore, PCR has been reported to identify noncultivable bacteria from

![Table 2. Performance of devR ‘long-length’ and ‘short-length’ assays in sputum](image-url)

<table>
<thead>
<tr>
<th>PCR assay and result</th>
<th>Culture positive (%)</th>
<th>USP smear positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gold standard</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>‘Long-length’ assay</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>202</td>
<td>19</td>
</tr>
<tr>
<td>Negative</td>
<td>71</td>
<td>214</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>73.99</td>
<td>69.87</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>91.85</td>
<td>96.9</td>
</tr>
<tr>
<td><strong>‘Short-length’ assay</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>269</td>
<td>52</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
<td>181</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>98.53</td>
<td>97.76</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>77.68</td>
<td>91.75</td>
</tr>
<tr>
<td>IS6110 (123 bp)†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>272</td>
<td>65</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>168</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>99.63</td>
<td>72.10</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>72.10</td>
<td>86.98</td>
</tr>
<tr>
<td>Total (n = 506)</td>
<td>273</td>
<td>233</td>
</tr>
</tbody>
</table>

*All culture-positive samples were USP smear positive.
†Data derived in part (IS6110 PCR and USP smear and culture data for the 506 specimens) from (Chakravorty et al., 2005) for ready reference.

USP, universal sample processing.
patients already under therapy till a period of 5–11 months after the first evaluation (Beige et al., 1995). In our study we excluded all specimens from patients on antituberculous therapy (ATT) at the time of specimen collection. The ‘false-positive’ specimens (considering USP smear as the gold standard) were from symptomatic patients presenting at the tuberculosis clinic, who were direct smear negative. As per RNTCP norms, this group of patients (direct smear negative) was not evaluated further and therefore information of their previous tuberculosis history or ATT status, if any, was not available to us. So, the apparent false positivity may be a reflection of very low bacterial loads not detected by USP smear or culture combined with the high sensitivity of the PCR assay.

Test efficiency is a measure of the reliability with which true positives as well as true negatives are detected accurately (Armitage, 1971). By this measure the ‘short-length’ and the IS6110 assays were both excellent, having efficiencies of 95.5% and 94.3%, respectively, in contrast to the ‘long-length’ assay whose efficiency was 80.2%. Taking into consideration the superior performance of the devR ‘short-length’ assay in the present study and keeping in mind that IS6110 elements are not universally present in clinical isolates in India, amplifying shorter fragments from devR, a gene which is universally present in clinical M. tuberculosis strains, would be very advantageous for the rapid and efficient detection of tuberculosis.

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References


