USE OF REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION FOR THE DETECTION OF MYCOBACTERIUM LEPRAE IN THE SLIT-SKIN SMEARS OF LEPROSY PATIENTS

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The relevance of bacterial index (BI) for understanding the prognosis of leprosy patients on treatment has been extensively debated, as it does not give a very clear idea of the viability of the bacteria in patients under treatment. Here we used slit-skin smear samples of leprosy patients to test the suitability for studying viability of Mycobacterium leprae using reverse transcription polymerase chain reaction (RT-PCR). For this purpose, we recruited 13 multibacillary (MB) leprosy patients (8 lepromatous and 5 borderline lepromatous). Of these, 7 were relapse cases, 3 were under treatment (MB-MDT), 2 were new cases and 1 had completed treatment. We carried out extraction of RNA using Trizol reagent (Life Technologies, UK) from the slit-skin smear samples from these patients. The RNA preparation was then used for the RT-PCR using Mycobacterium leprae-specific primers for the fragment of 16s ribosomal RNA gene. Samples from both the new cases, 4 suspected relapse cases and 1 patient under treatment showed positive RT-PCR results. Other 6 patients whose smear samples did not show any amplification by RT-PCR were on MB-MDT from 8 to 30 months. The usefulness of the technique needs to be validated using mouse footpad technique and also should be more extensively explored for studying the viability of M. leprae, the efficacy of treatment and the presence of other mycobacteria in the slit-skin smear samples.

INTRODUCTION

Leprosy is a chronic disease caused by Mycobacterium leprae and is still a public health problem in many countries. The introduction of MDT in the early 1980s has brought down the prevalence rate significantly. But some patients who had taken MDT treatment and who had been released from treatment (RFT) have relapsed (Haldar et al, 2003; Norman et al, 2004; Oliveira et al, 2002). Relapses have been reported in both MB and PB...
leprosy after successful MDT therapy (Cellona et al, 2003; Wu et al, 2002). Knowledge about persistent M. leprae or the multi-drug resistant M. leprae is very limited. The “resisters” escape or nullify the effect of drugs, whereas the “persisters” are sensitive to the drugs, but they reduce their metabolism to the minimum and are not acted upon by the drugs thus living in a dormant stage (Toman, 1981). M. leprae isolates obtained from referral relapse cases of leprosy showed viability after being tested for viability and drug sensitivity in the mouse footpad (Shetty et al, 2003). The emergence of dapsone (diamino-diphenyl sulphone, DDS)-resistant strains of M. leprae was reported as early as 1964 (Petit & Rees, 1964), then followed by the emergence of multidrug resistant strains (Matsuoka et al, 2003).

Detection of drug resistance and viability has been looked at by various methods. The morphological index (MI) is widely used to measure M. leprae viability. The MI had become the standard laboratory assay for monitoring the short-term bactericidal activity of new anti-leprosy drugs, but because of the lack of adequate standardization and subjective interpretation, the MI is difficult to apply. Conventional drug susceptibility testing of M. leprae from clinical specimens relies on the ability to cultivate M. leprae in the hind footpads of mice according to the method described by Shepard (1962). This method requires the recovery of sufficient number of viable organisms from a patient to inoculate the footpads. The results are available after six months to one year and, because of the need of large numbers of bacteria, patients with high bacterial loads only can be tested.

To reduce the number of organisms needed and to minimize the time required for drug susceptibility testing several protocols on genotype identification of mutants have been developed; these techniques are based on the amplification of specific DNA fragments from crude biological specimens using polymerase chain reaction (PCR)-based amplification (Santos et al, 2001; de Almeida et al, 2004). The PCR assays in leprosy have been useful in the detection of leprosy, assessment of efficacy of leprosy chemotherapy (Kampirapap et al, 1998) as well as mutations associated with drug resistance (Gillis & Williams, 1999). The RNA-based assays have been used in leprosy to look at the viability. Techniques such as reverse transcription (RT)-PCR (Katoch, 1998; Kurabachew et al, 1998) and nucleic acid sequence-based amplification (NASBA) targeting (van der Vliet et al, 1996), have been reported to be useful for the determination of viability of M. leprae. The 16s rRNA is targeted in many RT-PCR assays, but mRNA of the 18-kDa protein is also used as a target sequence (Chae et al, 2002). Detection of rRNA should impact increased sensitivity over assays based
on the detection of a single copy or even multiple copies of genomic sequences since each cell contains 1,000 to 10,000 copies of rRNA. An RNA-based detection method would be expected to better reflect the number of viable organisms because RNA generally degrades within a few minutes of cell death. Thus, an RNA-based detection system can be useful in determining the viability of M. leprae, which, in turn, could help to monitor the efficacy of treatment. Such approaches could also be helpful in differentiating conditions such as late reactions and relapses for patient care.

Various clinical samples like blood, nasal secretions (Santos et al, 2001), biopsies, slit-skin smears (Kamirapat et al, 1998) are used for the PCR assays, but for the RT-PCR assays mostly biopsies have been used so far. Here, in our study we used slit-skin smears from new cases, relapse cases and patients under treatment for RT-PCR and looked at its suitability, as well as tested for the methodology of RT-PCR so that it can be developed as a tool for determining the viability of M. leprae.

**METHODOLOGY**

**Patients**

13 patients visiting the Richardson Leprosy Hospital, Miraj, Maharashtra, India, were recruited for the study, after obtaining their written informed consent. All of them were of multibacillary (MB) type, classified as lepromatous (8) and borderline lepromatous (5) cases according to Ridley-Jopling classification (Ridley & Jopling, 1966). Details of all the patients are shown in Table 1. Treatment history of these 13 patients was as follows: 4 were dapsone monotherapy relapse, 3 were MB/MDT relapse, 3 were under MB-MDT treatment, 2 were new cases, and 1 was released from treatment. Dapsone monotherapy relapse amongst the four patients was reported after 12 years, 13 years, 25 years and 28 years. Relapse in the 2 MB-MDT cases was reported after 10 months and 8 years. There was one patient who was treated for both mono as well as multidrug therapy and then showed relapse after 8 years.
Slit-skin Smears

Fresh slit-skin smears were taken for microscopy and PCR. Slit-skin smears of some patients were collected successively as indicated in the results. The bacterial index (BI) as well as the morphological index (MI) of each smear was determined by microscopic examination. The BI, quantitative estimate of the bacteria on the basis of counting acid-fast bacilli, was obtained by employing Ridley’s logarithmic scale (Ridley, 1964). The MI of these subjects which denotes the percentage of solid bacilli (viable) to the total number of bacilli (Waters & Rees, 1962) was determined.

RNA Extraction

The scalpel blade from the smear with the tissue material on the tip was placed in a 1.5 ml sterile tube in normal saline. 800 µl Trizol (Life Technologies, UK) containing 200 mg glycogen (final cone, 250 mg/ml) was added, followed by vigorous vortexing or power homogenization. The sample was further vortexed, at room temperature, at high speed for 10 seconds ensuring that the Trizol reagent wets the side of the vial in order to solubilize any sample that may be remaining on the walls. The genomic DNA in the sample was sheared by passing twice through a 26-gauge needle connected to a 1 ml syringe. Using the syringe the sample was
transferred to a sterile 1.5 ml tube. 160 µl of chloroform was added to each sample and vortexed up to 30 seconds. After centrifugation at maximum speed the upper aqueous phase was transferred to a fresh tube and then 200 µl of ice-cold isopropanol was added. The samples were precipitated at -20 °C overnight. The RNA was pelleted by centrifugation at the maximum speed in the microfuge at room temperature. After decanting the supernatant, the pellet was washed in 200 µl of 70% ethanol and centrifuged again. The supernatant was decanted carefully without disturbing the pellet and the pellet was dried under vacuum. The pellet was resolubilized in 30-50 µl RNase-free deionized water.

We used 5 ml culture of Mycobacterium smegmatis (grown in Middlebrook’s 7H9 liquid medium) for the RNA extraction and subsequent RT-PCR as a positive control for the whole procedure.

RT-PCR

The RT-PCR protocol used was as described by Kurabachew et al (1998) using 16s-rRNA primers. The sequences of primers P1 and P3 were originally published by Arnoldi et al (1992), and the sequence of primer P2 was originally published by Cox et al (1991). The primers and their sequences were as follows: primer P1 - AGA GTT TGA TCC TGG CTC AG, primer P2 - TCG AAC GGA AAG GTC TCT AAA AAA TC, primer P3 - CCT GCA CCG CAA AAA GCT TTC C. The primers P1 and P3 amplified 227 bp fragment of 16s rRNA of any mycobacteria, and it was used for the amplification of Mycobacterium smegmatis containing the sample as well as Mycobacterium leprae. Primers P2 and P3 specifically amplified 173 bp fragment of 16s rRNA of M. leprae. The RT-PCR was carried out by using ready-to-go RT-PCR beads obtained from the Pharmacia Biotech. The PCR amplification was performed in 50 µl reaction volumes containing 10 mM Tris-HCl (pH 9.0 at room temperature), 60 mM KCl, 1.5 mM MgCl₂, 200 µM deoxynucleoside triphosphates. 50 ng each of primers P2 and P3, 2.0 U of Taq DNA polymerase, 10 µl of sample (template), Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase, ribonuclease inhibitor (porcine) and stabilizers, including Rnase/DNase-free BSA with a Hybaid Omnigene thermal cycler. The negative control was set up to see DNA contamination. Ready-to-go RT-PCR bead was suspended in 40 µl of primer mix after which reverse transcriptase enzyme was denatured at 94°C for 10 minutes to which 10 µl of sample was added. A negative control was set up for all individual samples. The cycling profile for the two sets of 16s rRNA primers involved reverse transcription at 42°C for
50 minutes followed by PCR consisting of 37 cycles of denaturation at 94°C for 2 minutes, annealing at 60°C for 2 minutes, and extension at 72°C for 3 minutes, followed by final extension at 72°C for 10 minutes.

**Agarose Gel Electrophoresis**

The amplified RT-PCR product was identified by using agarose gel electrophoresis. 5 μl of the RT-PCR product along with the Orange G (10 mg/ml) loading dye was electrophoresed through 1.5% agarose gel prepared in Tris-borate EDTA (TBE) buffer containing ethidium bromide (10 mg/ml) dye at a final concentration of 0.5 μg/ml at 70 V for 2 hours.

**RESULTS**

**Bacterial Index**

All the 13 patients selected were having high BI (Table 1), the highest average BI being 5.6+ and the lowest being 2.3+. Out of the 13 patients included in the study, only 2 were new cases and others were relapsed cases.

**Morphological Index**

At the time of smear collection for RT-PCR none of the patients showed solidly stained bacilli (0% MI). But the records showed that two of the patients (patients no. 1 and no. 3) had shown 2% and 1.1% MI respectively at the start of treatment. The second patient (patient no.3) had shown solidly stained bacilli only in the smear from the back. But both these patients, at the time of smear collection for RT-PCR, had become negative for MI.

**RT-PCR**

RT-PCR using *M. smegmatis* sample showed amplification product of 227 bp (Fig. 1A). We could also see the amplification in 7 samples of patients with primers P1 and P3. The same smear sample when amplified with P2 and P3 primers showed 173 bp product (Fig.1B). None of the negative control samples showed any amplification where denaturation of reverse transcriptase was done prior to amplification, i.e. without allowing reverse transcription process for the conversion of RNA into cDNA.
Fig 1. Agarose gel electrophoresis of RT-PCR products

1A shows amplification products (227 bp) obtained with primers P1 and P3.

1B shows amplification products (173 bp) obtained with primers P2 and P3. Marker lane was loaded with OX174DNA/HaeIII fragments.

All the slit skin-smears, as mentioned above, were subjected to RT-PCR using P2 and P3 primers and the agarose gel electrophoresis and the results noted: 7 patients out of the 13 showed positive RT-PCR results, as shown in Table 2.

Table 2. Results of RT-PCR

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>RT-PCR</th>
<th>BI average</th>
<th>Recent history of treatment (at the time initial smear collection for RT-PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>4+</td>
<td>12 MB – MDT doses</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>4.25</td>
<td>8 MB – MDT doses</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>0 (4-back)*</td>
<td>13 MB – MDT doses</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>5+</td>
<td>24 MB – MDT doses</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>3+</td>
<td>30 MB – MDT doses</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>2.3+</td>
<td>24 MB – MDT doses</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>5.5+</td>
<td>Nil</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>4+</td>
<td>8 doses</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>5.6+</td>
<td>Nil</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>4+</td>
<td>Nil</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>3+</td>
<td>Nil</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>4+</td>
<td>Nil</td>
</tr>
<tr>
<td>13</td>
<td>+</td>
<td>3.33+</td>
<td>1 dose</td>
</tr>
</tbody>
</table>

* One lesion on the back had a BI of 4+. Regular smears sites had a BI of 0. Smear collected from the back for RT-PCR.
Out of the 7 RT-PCR positive patients, 5 had not yet received treatment for relapse and two were under treatment—of these two, one received 20 doses and the other received only 1 dose. The PCR negatives were all treated for relapse with MB-MD'T, ranging from 8 to 30 doses.

Four patients (three with RT-PCR positive result and one with negative result) were successively followed up and slit-skin smears at intervals were collected for RT-PCR. As shown in Table 3, it was observed that 3 patients (patients no. 7, 8 and 10) who were initially RT-PCR positive remained positive in the subsequent follow-up at different time points. The patient for whom the initial RT-PCR result was negative (patient no. 5) remained negative in the subsequent follow-ups.

| Table 3. Results of follow-up of patients for RT-PCR |
|---------------------------------------------|---|---|---|
| Duration after start of treatment | BI | RT PCR |
| Patient No. 7 | 0 days | 5.5+ | + |
| Patient No. 7 | 2 days | Not done | + |
| Patient No. 7 | 14 days | Not done | + |
| Patient No. 7 | 28 days | Not done | + |
| Patient No. 7 | 4 months | Not done | + |
| Patient No. 8 | 8 months | 4+ | + |
| Patient No. 8 | 22 months | 4+ | + |
| Patient No. 10 | 0 | 4+ | + |
| Patient No. 10 | 2 months | Not done | + |
| Patient No. 5 | 27 months | 3+ | — |
| Patient No. 5 | 32 months | 3.5+ | — |
| Patient No. 5 | 34 months | 2.75 | — |

DISCUSSION

Testing of viability of *M. leprae* is very important to determine the efficacy of treatment. Most widely used mouse footpad assay has a major drawback as it takes more than 6 months to give the results, but still it is the only gold standard available for testing viability. Various researchers have used PCR for the diagnosis of leprosy using various samples, such as skin biopsies, nasal secretions, blood, etc. (Santos et al., 2001; de Almeida et al., 2004; Kampirapap et al., 1998). PCR has also been used to look at the efficacy of chemotherapy (Kampirapap et al., 1998) and drug resistance (Gillis & Williams, 1999). The detection of 16s rRNA of *M. leprae* may
correlate to the viability of *M. leprae* as RNA degrades soon after the cell death, which means RNA will not be detected in the cells that are dead. Biopsy material is used to look at the viability by some researchers (Kurabachew *et al.*, 1998; Chae *et al.*, 2002) by RT-PCR. Biopsy may not be an easily available clinical material due to various technical and ethical reasons. In our study we wanted to look at the suitability of slit-skin smears as sample for RT-PCR assay as well as to test the methodology of RT-PCR. RT-PCR amplification, using *Mycobacterium smegmatis*, as well as the use of negative controls clearly showed that by using the method described above we do not get any DNA contamination in the RNA extracted, and also the reverse transcription as well as amplification works very well.

From the results obtained from clinical samples, it is evident that some patients showed RT-PCR positivity, indicating that the RNA extracted from the slit-skin smear contained 16s rRNA of *Mycobacterium leprae*. Out of 7 positive samples, 5 samples were from patients who had not yet taken any treatment, and one who had just started the treatment (1 dose). Thus, the six patients with positive RT-PCR results were likely to have viable bacilli in their body. But surprisingly for one patient (patient no. 8), in spite of being on MB-MDT treatment for considerable time, the RT-PCR result was positive. The negative results that were observed were mostly in those patients who were taking MB-MDT treatment for relatively longer time (8-30 doses). This would suggest that most of the viable *M. leprae* had been killed due to the treatment.

In our study 4 patients were followed up successively. One patient (patient no. 7) was found to be RT-PCR positive in all the five tests that were performed on the samples collected from him. Another patient (patient no. 8), as mentioned above, even after 22 months (20 doses of MB-MDT) was RT-PCR positive. This may point towards drug resistance, but we were unable to take successive samples as we lost the patient in the subsequent follow-up.

The MI results showed the absence of solid (viable) bacilli in all the smear samples, but RT-PCR results suggest that we really cannot exclude the presence of viable bacilli in the smears. Dhopale (1984) and Katoch *et al.* (1988) have shown that MI is a less sensitive index of viability than ATP content of *M. leprae*. When MI is 1% or less there may still be some viable bacilli which are missed because of clumping of bacilli or because of sampling error.
Hellyer et al (1999) showed that mRNA-targeted RT-PCR is a more sensitive tool for *Mycobacterium tuberculosis* for viability studies. But due to highly labile nature of mRNA, the extraction procedure for clinical samples becomes very difficult. Moore et al (1996) reported that there is no extended rRNA carrier state in pulmonary tuberculosis patients who have successfully completed treatment. But there was a non-cultivable organism shedding period where RT-PCR results for rRNA were positive, but culture tests were negative. Not much is known as to how long ribosomal RNA from *M. leprae* will persist after the death of the cells. But it is unlikely that the RNA will persist for longer than a few days. van der Vliet et al (1994) have shown that nucleic-acid sequence-based amplification (NASBA) targeting 16s rRNA in *Mycobacterium smegmatis* could be a potential tool for rapid evaluation of drug susceptibility testing and also rRNA decays rapidly after cell death. Similarly, Bentsink et al (2002) have also shown that NASBA allows direct detection of viable cells of *Ralstonia Solanacearum* (causal organism for bacterial wilt in potato). Detection of rRNA could be a good and early index of the metabolic activity of the microorganisms.

As the slit-skin smears are easy to collect and easily available, they will be quite useful for the purpose of testing viability of *M. leprae* by using RT-PCR. The results here clearly show that the methodology works quite well with simplified RNA extraction procedure, such as Trizol extraction. Nevertheless, as mouse footpad assay is the only accepted gold standard available for viability studies of *M. leprae*, it is important that the results of RT-PCR are validated with this technique. This is important, especially for the RT-PCR-negative samples, to ensure that negative samples do not have viable bacilli. Furthermore, this study was done with multibacillary patients just to check the protocols and suitability of the samples. We need to see the sensitivity of the method using paucibacillary patients. Also, it is important to identify mRNA targets for the amplification, as their half-life is much shorter than rRNA, which, in turn, can better reflect the viability.

**REFERENCES**


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