Analysis of gene probes and gene amplification techniques for diagnosis and monitoring of treatment in childhood leprosy

RAJ KAMAL*, R. DAYAL**, V. M. KATOCH* & K. KATOCH*

*National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Tajganj, Agra, India
**Department of Pediatrics; S.N. Medical College, Agra, India

Accepted for publication 15 February 2006

Summary Nucleic acid sequences of Mycobacterium leprae were detected using gene probes hybridizing with targeting ribosomal RNA (16S rRNA), ribosomal DNA (16S rDNA) and gene amplification techniques (PCR) in skin lesion of paediatric leprosy patients and the effect of treatment on the by these methods. Eighty paediatric leprosy patients were included in the study. Most cases (79%) were between 9 and 16 years of age. Cases were divided into three groups according to treatment status, viz. untreated (30), undergoing treatment (30), and at the end of treatment (20). Clinical and slit smear examination for acid fast bacilli (AFB) was performed and nucleic acids were extracted and fractionated from skin biopsies. *M. leprae* specific 16S rRNA and 16S rDNA was detected by hybridization with gene probes whereas the 36 kDa gene sequence was detected by a gene amplification assay (PCR). The cases were classified as paucibacillary (PB) and multibacillary (MB) by the standard criteria of WHO (1988). Positivity of 16S rRNA in PB cases decreased from 60% in untreated to 10.5% after 4–8 months of treatment whereas for 16S r DNA, it decreased from 50% to 21%, for PCR from 70% to 36.8% for the same specimen, and all became negative at 1 year. Similar trends were seen in MB cases where positivity in smear positive untreated cases decreased from 100% to 56.2% with 16S rRNA and 42.8% with 16S rDNA and PCR, respectively, after 9–12 months of therapy and all became negative at 2 years, except one case which remained positive with PCR. Similar results were observed in smear negative MB cases, 100% positivity detected by 16S r RNA and PCR, 75% detected by 16S rDNA decreased to zero after 9–12 months of therapy. This study suggests the potential usefulness of gene probes targeting 16S rRNA and 16S rDNA and PCR as supportive molecular tools for diagnosis of smear negative evolving MB disease and also monitoring the response to treatment, these observations however, needs to be validated in prospective follow up studies.

Correspondence to: R. Kamal (e-mail: rohinik@sancharnet.in)
Introduction

A major proportion of the leprosy cases remaining in the world reside in India and a few other countries such as Brazil. For early eradication of the disease, it is important that these are diagnosed early and treated so that the deformities can be prevented and leprosy can be effectively controlled. Accurate diagnosis is important in children. Conventional methods of diagnosis include characteristic clinical findings, demonstration of AFB and histopathological examination. However, in early and suspected cases, the diagnosis may be doubtful by using these traditional methods, and cases have to be followed up for varying periods till a definite diagnosis can be made. Such cases need alternative objective tests for confirmation. The application of recently developed gene probes and gene amplification methods which are highly sensitive and specific, thus assumes great importance in the early diagnosis of these cases.

Recently, several gene probes and gene amplification techniques for detection of *Mycobacterium leprae* nucleic acids have been developed. Application of r-RNA and r-DNA targeting probes had shown their potential in confirmation of diagnosis of leprosy in children. Also, the polymerase chain reaction (PCR) has provided additional capabilities in confirming the diagnosis of disease. These gene amplification techniques can detect the specific presence of *M. leprae* nucleic acids even from specimens with 1–10 organisms. There is inadequate information about the persistence/disappearance of these nucleic acids after treatment which is clinically and therapeutically relevant. This study reports these observations detection of 16S rRNA, 16S rDNA, and amplification of 36 kDa gene targets from biopsy specimens of children at different stages of treatment.

Materials and methods

This study was undertaken between January 2001 to March 2004 at the Department of Medicine (Medical Unit 1) and Microbiology & Molecular Biology, Central JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra and Department of Pediatrics, S.N. Medical College, Agra.

The research approval for the study was taken from Scientific Advisory Committee of the Institute and the study is also monitored by the same committee yearly. Ethical approval was taken from Ethical Committee of the Institute on 14 October 1998 as part of a project entitled ‘Probes for early diagnosis and possible strain differentiation’; since then, it has been reviewed periodically.

Eighty children, below 16 years of age who were at different stages of treatment were included in the study after informed consent was obtained from their parents. A case of leprosy was defined as a person showing clinical signs of leprosy with or without bacteriological confirmation of the diagnosis by demonstration of acid fast bacilli (AFB) in the slit skin smear. These cases were classified using the standard criteria of the WHO Expert Committee on Leprosy, Geneva 1988. Paucibacillary (PB) leprosy included only smear negative (Indeterminate-I, Tuberculoid-TT, Borderline Tuberculoid-BT) cases, whereas multibacillary (MB) leprosy included smear positive Borderline Tuberculoid-BT and other cases of Borderline-BB, Borderline Lepromatous-BL and Lepromatous-LL.

In this study, the patients were also classified into three groups on the basis of their treatment status: those who had not received any treatment (untreated), those who were undergoing treatment (4–8 months for PB cases and 9–12 months for MB cases), and those
who were at the end of treatment (1 year for PB and 2 years for MB cases, respectively). All
the patients were treated with regimens either standardized or being used at the Institute.8,9
These patients were regularly followed up while on treatment.

The treatment regimen for MB cases (over 10 years) contained rifampicin 450 mg (once a
month), clofazimine 50 mg on alternate days and dapsone 50 mg daily. For children under 10
years the dose was adjusted to rifampicin 300 mg (once a month), clofazimine 50 mg twice a
week, and dapsone 25 mg daily was given up to 2 years.8

The treatment regimen for PB cases (above 10 years) contained rifampicin 450 mg once a
month and dapsone 50 mg daily for 1 year. For children under 10 years, the doses were
adjusted to rifampicin 300 mg and dapsone 25 mg. Treatment was stopped at the end of 1
year.9 A detailed history, thorough clinical examination and slit smear for AFB, was taken.
Skin biopsies were taken from active lesions of these patients.

EXTRACTION AND FRACTIONATION OF RNA AND DNA

Skin biopsies collected from the patients were homogenized in TE buffer (Tris-EDTA, pH 8).
Nucleic acids were extracted, fractionated into rRNA and rDNA by an integrated
physiochemical procedure comprising of freezing–thawing, sequential enzymatic treatment
with lysozyme and proteinase k following by fractionation by precipitation with 0·75 v/v
ethanol for RNA and subsequently 0·75 v/v isopropanolol for DNA after suspension in lysis
buffe.10 Residual contamination of DNA in rRNA preparation was eliminated by treatment
with DNAs (Sigma).

PROBES

The following two probe targeting different stretches of ribosomal RNA gene region of
M. leprae designed by Katoch et al.2,10 and as used by Sharma et al.11,12 and Dayal et al.4,5
were used in the study: (i) 17-mer synthetic DNA oligonucleotide probe 5′ CACTGGGCTCC
TTCAAGGCGGATGTCTT-3′, against a stretch of 1425–1441 position of 16S rRNA, and (ii) 18-mer
oligonucleotide probe 5′ CTTCAAGGCGGATGTCTT-3′ targeting the 192–209 region of
16S rRNA.

Probes were procured from Bioserve and Biotechnology (Hyderabad, India) and were 3-
end-labeled with DIG using kit no. 3353575 (Roche Diagnostics, Germany). Fractionated
ribosomal RNA and DNAs were blotted on nylon membrane and fixed in a oven at 120 °C for
30 min (Gallenkamp, UK). Hybridization with probes was done using the procedure
described earlier.12,13

PCR

A 530 bp fragment encoding part of 36 kDa gene was amplified by the procedure, described
by Hartskeerl et al.3,6 in a thermal cycler (M. J. Research USA, Model PTC 100). The
identities of amplicons were confirmed by gel electrophoresis and southern blot hybridization
using a DIG labeled probe.

Results

Eighty cases were studied, most (79%) aged 9–16 years. The majority of patients had
hypopigmented (74-75%) and macular (63-75%) lesions present on covered as well as
uncovered body areas. Cutaneous sensation was affected in most patients, while nerve thickening was observed in 75% cases. Skin smear examination showed that 91% of PB cases (total 41) were smear negative while 9% were smear positive (Table 1). The majority of MB cases (66·25%) were smear positive.

**PAUCIBACILLARY SPECIMENS**

Biopsies from 60% of untreated cases were positive for 16S rRNA, 50% were positive by probes hybridizing with targeting 16S rDNA, while 70% were positive with PCR. In patients undergoing treatment (4—8 months), 10·5%, 21%, and 36·8% were gave positive results with probes hybridizing with targeting 16S rRNA, 16S rDNA, and PCR, respectively. No positive signal was detected after 1 year of treatment in these cases with either probes or PCR (Table 2).

**MULTIBACILLARY SPECIMENS**

Among the untreated group all smear positive cases were positive for 16S rRNA, 16S rDNA and with PCR. In the smear negative group, 100% positivity was observed with probes hybridizing with targeting 16S rRNA and with PCR while 75% cases were positive with probes hybridizing with targeting 16S rDNA.

In MB patients undergoing treatment, 42·8% smear positive cases had positive signals while probes hybridizing with targeting 16S rDNA and with PCR while all smear negative cases were negative with both probes and with PCR. None of the smear negative case towards the end of treatment had positive signals, only one smear positive case was PCR positive in this group (Table 3).

**Discussion**

In the absence of demonstrable AFB, the diagnosis of leprosy is based on clinical signs and histopathology which may be ambiguous in a proportion of cases, particularly in those presenting with hypopigmented and macular skin lesions; such cases were in majority in present series and similar profile have been reported by other workers in leprosy in children. Macular forms are considered to be the early form of the disease and have been reported more commonly in India.4,5,15,16 Not only may these lesions sometimes pose a diagnostic problem, but the disease activity may also be difficult to monitor.

**Table 1. Slit skin smear examination results in patients recruited to the study**

<table>
<thead>
<tr>
<th>Length of treatment</th>
<th>Smear results</th>
<th>I</th>
<th>TT</th>
<th>BT</th>
<th>BB</th>
<th>BL</th>
<th>LL</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>Positive</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>4–8 months &amp; 9–12 months treatment</td>
<td>Positive</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>2</td>
<td>4</td>
<td>13</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>23</td>
</tr>
<tr>
<td>1–2 years of treatment</td>
<td>Positive</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>5</td>
<td>6</td>
<td>30</td>
<td>19</td>
<td>15</td>
<td>5</td>
<td>80</td>
</tr>
</tbody>
</table>
We observed that not only could rRNA and r DNA be detected by probes and *M. leprae* specific sequences by PCR in untreated MB and PB cases but these molecular tools can also monitor the progress under treatment. Positivity is much higher in MB cases and a significant proportion of smear negative MB type are positive. Ribosomal DNA detection was found to be less sensitive in PB cases. As in earlier studies, these probes gave positive results in all smear positive untreated cases and in a significant proportion of smear negative cases. Though not statistically significant, the overall sensitivity achieved with these probes in PB cases alone appear to be inadequate and is lower than that observed with PCR. This data shows that these probes and PCR can be used only as supplementary techniques and when negative clinician will have to rely on clinical and histological correlates.

Higher positivity by probes targeting 16S rRNA in untreated cases as compared to 16S rDNA targeting probes can be explained by the DNA targeting probes requiring $10^4–10^5$ targets for a positive signal, whereas ribosomal RNA targeting probes have a larger copy number per live cell (2000–5000 copies) and these degrade faster after bacterial death.\(^2,10,11,12\) Such an advantage was not statistically significant in the current series, possibly because of the low number of cases. The benefits of these applications need to be analysed and statistically proven in a significant number of cases. This study also shows that 16S rRNA positivity decreases after treatment, Thus the detection of 16S rRNA can be used for monitoring treatment which is expected to correlate with loss of viability of *M. leprae* in leprosy cases. A decrease in signals has been reported in MB adult cases under treatment,\(^12\) but experience in less bacillated smear negative cases has been limited. While the findings need to be validated in a larger number of cases, trends are promising. The proportion of cases with detectable nucleic

<table>
<thead>
<tr>
<th>Table 2. Nucleic acid probe and PCR results for PB patient biopsies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Probe</strong></td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Length of treatment</strong></td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>Untreated</td>
</tr>
<tr>
<td>4–8 months of treatment</td>
</tr>
<tr>
<td>1 year of treatment</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 3. Nucleic acid probe and PCR results for MB patient biopsies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Length of treatment</strong></td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>Untreated</td>
</tr>
<tr>
<td>9–12 months of treatment</td>
</tr>
<tr>
<td>After 2 years of treatment</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
</tbody>
</table>
acid sequences progressively decreased. As reported earlier, DNA signals detected by PCR decrease with treatment, but may persist in a proportion of cases,17,18 As the recommended treatment duration has been further reduced and is nearly in the same range as cases chosen in middle of therapy in the present series, the significance of these positive signals at such duration needs to be determined in follow-up studies.

To conclude, this study supports the potential usefulness of hybridization with gene probes targeting 16S rRNA or 16S rDNA for confirming active MB disease, and it may be also used for monitoring the treatment. For maximum sensitivity, the use of gene amplification methods such as PCR (possibly by targeting m-RNA or r-RNA as target) may be required.

Acknowledgements

The authors are thankful to the paramedical and laboratory staff for their technical assistance and to LEPRA UK, for their valuable gifts of some of reagents.

References