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RESEARCH ARTICLE

Mycobacterium leprae 18-kDa heat shock protein gene is polymorphic

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Leprosy is still a major health problem in India. *Mycobacterium leprae*, the organism causing the disease, appears to be genetically invariant. Attempts to identify strain variation in different isolates of *M. leprae* have met with little success. The *M. leprae* 18-kDa heat shock protein is a major T-cell antigen, and this protein belongs to the family of small heat shock proteins. In the present study, we have analysed the mRNA expression profiles of *18-kDa* gene in lesions across the leprosy spectra by RT–PCR and also sequenced the PCR amplicons prepared from 25 independent leprosy cases. A single nucleotide polymorphism was detected at 154th bp position in this secreted antigen gene. In this gene, codon 52 exists as TCA in about 60% of the samples and as CCA in rest of the leprosy cases. Armadillo-derived *M. leprae 18-kDa HSP* gene has TCA at the 52nd position. Among the cases examined, we could not detect mixed population of bacteria carrying both classes of genes, indicating the clonal purity of the bacterium in an infected patient. This polymorphism therefore, could be used as a molecular marker in strain typing of *M. leprae* in endemic populations. In addition, transmission patterns and relapse and/or reinfection in reactional patients could be examined using this polymorphism. Expression of the *18-kDa HSP* gene in reactional cases indicates that live bacterium might be contributing to the reactional conditions in leprosy.

LEPROSY is a chronic infectious disease caused by the acid-fast bacillus *Mycobacterium leprae*. Leprosy is still a health problem globally, and India has 64% of the global leprosy burden[1]. Leprosy is an immunological disease with defined immunological and clinical parameters, and ranges from tuberculoid (TT) to lepromatous (LL) leprosy with a range of borderline cases between the polar ends[2]. TT patients have robust, cell-mediated immune response that restricts the growth of the pathogen leading

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to the clearance of infection. LL patients have a predominant humoral response and are anergic to *M. leprae* antigens[2].

The borderline tuberculoid (BT), mid borderline (MB) and borderline lepromatous (BL) leprosy patients are unstable immunologically and they might convert to the next level. A considerable proportion of borderline patients (20–30%) develop acute immunological reactions during the course of the disease in the form of reversal reactions (RR or type-I reaction) or erythema nodosum leprosum (ENL or type-II reaction)[3]. BL and LL patients undergo ENL reaction and ENL is associated with severe tissue damage mediated by the deposition of immune complexes and complement activation[4].

Strain-typing methods for *M. leprae* would be of great value to identify the source of infection, to understand the transmission patterns of the organism and to identify relapse and reinfection in patients, especially in reversal reactions[5]. Several attempts have been made to identify polymorphic DNA sequences which could be used to identify *M. leprae* strain differences[6–9]. However, RFLP analysis using various probes has not shown any differences among *M. leprae* isolates[6–9]. Further, single-strand conformation polymorphism (SSCP) of DNA sequences encoding 16S and 23S rRNA was also identical in *M. leprae* isolated from different multibacillary leprosy patients[10]. A recent report on *M. leprae*-specific repetitive sequences, RLEP, suggested variability among *M. leprae* isolates, since PCR amplification of this repeat showed different intensities and the RLEP sequence was absent in certain *M. leprae* isolates[11]. A majority of high-level, dapsone-resistant *M. leprae* strains was found to contain mutations in either codon 53 or 55 of the *folP1* gene[12], and multiple mutations were reported in the *rpoB* gene resulting in rifampicin resistance[13]. The utility of these mutations in strain identification is not yet attempted. Based on the number of TTC repeats found in *rpoT* gene, *M. leprae* strains have recently been classified into two subtypes[5].

The 18-kDa HSP antigen of *M. leprae* belongs to a family of small heat shock proteins[14] and is reported to be a major T-cell antigen[15–17]. The gene encoding this *HSP* gene has been cloned and sequenced[14]. The *18-kDa* antigen gene is highly specific for *M. leprae*[18], although genes with limited sequence homology were reported in *M. avium*, *M. scrofulaceum*, *M. gordonae*, *M. chelonei*, *M. intracellular* and *M. habana* based on Southern hybridization analysis[19–21]. However, the probe designed to target *18-kDa* antigen gene did not hybridize to human, murine or armadillo DNA[22]. Hence, this gene has been used as a target for several studies for detection of *M. leprae* from leprosy samples by PCR[23–26] and RT–PCR[27]. The epitopes of this heat shock protein have been well characterized[28,29]. T-cell clones specific for peptides carrying amino acids 1–38 and 41–55 were found to cross react with the *18-kDa* antigen of *M. tuberculosis* complex, *M. avium* and *M. scrofulaceum*. However, T-cell clones specific for peptides carrying amino acids 38–50 show only limited cross reactivity[30].

In this study we report a single nucleotide polymorphism (SNP) in the *M. leprae 18-kDa HSP* gene from leprosy patients examined across the leprosy spectrum. The patients came from the same geographical region in Tamil Nadu. Reverse transcription PCR of mRNA of *18-kDa* antigen was used to clone the gene using paraffin-embedded biopsy material following a method described by us earlier[31]. The present study shows that the 154th base exists either as T or C and consequently the 18-kDa HSP exists as two classes of protein, one with serine at 52nd amino acid (class I) and the other carrying proline at this position (class II). In addition, we have also demonstrated the expression of mRNA in 11 out of 14 reactional cases, which implies that pathogen-specific genes might play a role in reactional conditions.

Materials and methods

Specimen collection and bacterial index

Leprosy patients were classified with respect to the spectrum of leprosy, according to the clinicopathological classification of Ridley and Jopling[2]. Histopathological studies of similar cases done earlier confirm clinicopathological data (unpublished results). Punch biopsies were collected from patients after obtaining the informed consent according to the norms laid down by the institutional ethical committee and Indian Council of Medical Research at the Leprosy Hospital, Voluntary Health Services, Shakthi Nagar, Tamil Nadu. This study includes forty-six patients across the leprosy spectrum: TT ($n = 4$), BT ($n = 8$), BL ($n = 9$), LL ($n = 11$), type-I reaction or RR ($n = 7$) and ENL reactions ($n = 7$). The bacterial load in each biopsy sample was determined by acid-fast staining and expressed on a logarithmic scale as bacterial index (BI) (ref. 32) (Table 1).

Preparation of RNA

Biopsy materials were fixed in buffered formaldehyde and embedded in wax. Five micron sections of the PET were deparaffinated and RNA was extracted as already reported[32]. In brief, sections were deparaffinated by incubation in octane followed by ethanol wash. The samples were incubated in 200 µl of digestion buffer (10 mM Tris HCl, 25 mM EDTA, 100 mM NaCl, 0.5% SDS, Proteinase K at a final concentration of 300 µg/ml (Sigma, St. Louis, Missouri, USA) at 52°C with constant shaking for 24 h followed by extraction with 1 ml of Trizol (Gibco BRL, Grand Islands, New York, USA). Transfer RNA (Sigma; 10 µg) was used as carrier and RNA was precipitated by overnight incubation at – 20°C. RNA was

pelleted by centrifugation at 12,000 rpm for 30 min at 4°C and the RNA pellet was washed with 75% ethanol and dried under vacuum. RNA pellet was dissolved in 20 µl of DEPC-treated water. Approximate concentration of the total RNA was assessed spectrophotometrically.

Preparation of cDNA

Samples were treated with 10 U of RNAse free DNAse (Promega, Madison, USA) followed by reverse transcription carried out using oligo dT primers in a reaction mix containing the following: 1 µl of oligo dT_{15} primer (Promega; 0.5 µg/µl), 0.5 µg of RNA, 2 µl of DEPC-treated water (Sigma). The reaction mixture was incubated at 70°C for 10 min and chilled on ice. At the end of the incubation period first strand buffer (4 µl; 5 X buffer containing 250 mM Tris–HCl pH 8.3, 375 mM KCl and 15 mM $MgCl_2$), 2 µl DTT (100 mM stock), 1 µl of RNAsin (40 U/µl) (Promega), 1 µl of dNTPs (10 mM each) and 200 U of MMLV reverse transcriptase enzyme (Gibco BRL, Gaithersburg, USA) were added and incubated at 37°C for 1 h. The enzyme was heat inactivated at 92°C for 2 min and the cDNA was stored at – 20°C.

Table 1. Clinical spectra of patients from whom biopsy samples were obtained

S. No.	Sex	Disease status	Bacterial index
LL			
1	M	MB/LL	4 +
2	M	MB/LL	3.7 +
3	M	MB/LL	5 +
4	M	MB/LL	4.2 +
5	M	MB/LL	4.3 +
6	M	MB/LL	3 +
7	M	MB/LL	5 +
8	F	MB/LL	5.3 +
9	F	MB/LL	5 +
10	F	MB/LL	4 +
11	M	MB/LL	6 +
BL			
12	M	BL	–
13	M	BL	5 +
14	M	BL	4 +
15	M	BL	3 +
16	F	BL	1 +
17	M	BL	–
18	M	BL	3 +
19	M	BL	5 +
20	M	BL	1 +
BT			
21	M	BT	–
22	M	BT	–
23	F	BT	0.25 +
24	M	BT	0.45 +
25	M	BT	–
26	M	BT	–
27	F	BT	–
28	M	BT	0.25 +
TT			
29	M	TT	–
30	F	TT	–
31	M	TT	–
32	M	TT	0.7 +
RR			
33	M	BT/RR	–
34	M	BL/RR	4 +
35	F	BB/RR	3 +
36	M	BB/RR	–
37	F	BL/RR	1.75 +
38	M	BL/RR	3.7 +
39	M	BL/RR	2.7 +
ENL			
40	F	LL/ENL	4 +
41	M	BL/ENL	2 +
42	M	LL/ENL	3 +
43	M	BL/ENL	2 +
44	F	LL/ENL	2 +
45	M	LL/ENL	4 +
46	M	LL/ENL	3 +

PCR amplification of cDNA

PCR amplification of cDNA templates was carried out using MJ Research Thermocycler for 30 cycles with the following cycling parameters: denaturation at 94°C for 1.5 min, annealing at 54°C for 1 min, extension at 72°C for 1.5 min for 30 cycles followed by a final extension of 7 min. The primers sequences are β-actin: Forward primer 5′GTGGGGCGCCCCAGGCACCA and reverse primer 5′CTCCTTAATGTCACGCACGATTTC; *M. leprae* 18-kDa: Forward primer 5′ATGCTGATGCGTACTGACCC and reverse primer 5′TTAGGCATCTATGATTTCGT. The *18-kDa* HSP gene primer was designed to target the full-length gene. Amplification of human β-actin mRNA was used to normalize the amount of template and the efficiency of PCR reaction. β-actin mRNA primers flank an intron sequence and hence the PCR product would be 1112 bp if the template is contaminated with chromosomal DNA as against 540 bp, if cDNA is the template. PCR was performed in a 25 µl reaction mix containing 2 µl of cDNA, 1 U of Taq DNA Polymerase (Gibco BRL) (1 mM of $MgCl_2$ and 100 µM of dNTPs). Semi-quantitative PCR analysis was carried out by terminating PCR reactions at 18, 22, 26 and 30 cycles. PCR products were electrophoresed on a 2% agarose gel and the bands were visualized after staining with ethidium bromide. Template concentration in the samples was adjusted such that the intensity of the β-actin amplicon at 18, 22, 26 and 30 cycles in different samples was identical and the increase in the amplicon concentration is linear up to 30 cycles. Grading of signals as + 1 to + 4 was done depending upon the number of cycles after which a detectable PCR amplicon could be found, as described earlier[31].

Purification of PCR products for sequencing

PCR products were purified before sequence analysis. In brief, 8 µl of 5 M NaCl, 8 µl of TE (pH 8.0) and 14 µl of 40% PEG 8000/10 mM $MgCl_2$ were added to a 1.5 ml microcentrifuge tube and vortexed briefly. Then 50 µl of the PCR product was added to the above mixture and

vortexed and incubated at room temperature for 20 min. The mixture was centrifuged at 12,000 rpm for 15 min followed by two washes of the pellet with alcohol. The pellet was air-dried and dissolved in 15 µl of sterile water. Next 2 µl of the PCR product was electrophoresed on a 2% agarose gel to check the quality of the amplicon.

Sequencing of PCR products

All the sequencing reactions were carried out with an automated DNA sequencer (ABI Prism 377, PE Applied Biosystems) using Big Dye terminator kit (Perkin–Elmer, USA) at the DST–FIST DNA sequencing facility at School of Biotechnology, Madurai Kamaraj University, Madurai.

Sequence analysis

The sequence comparisons were performed using BLAST program from the National Center for Biotechnology Information World Wide Web server. Default settings were used with these programs.

Results

18-kDa HSP gene is expressed across the spectrum of leprosy cases

Semi-quantitative RT–PCR analysis of *18-kDa* HSP mRNA expression showed that the mRNA level was highest in LL cases. Although the expression is less, the mRNA was detectable in TT biopsy samples as well. Surprisingly, the levels were not uniform in lesions of different individuals belonging to the same clinical type of leprosy (Figure 1). In addition, the *18-kDa* antigen-specific mRNA was not detectable in one case of BL and two cases of BT. The mRNA could be detected even in those cases where the bacteria could not be detected under the light microscope using acid-fast staining. Low levels of *18-kDa* gene were detected in all ENL cases, except two cases where no amplification was observed (Figure 1). In all cDNA samples, β-actin was amplified in order to normalize the amounts of cDNA taken for SQ–RT–PCR across samples (Figure 2, lanes 1–4). A representative gel showing the amplicon of the *18-kDa* antigen gene is shown in Figure 2 (lanes 5–8).

18-kDa HSP mRNA is not detected in non-leprosy skin diseases

In order to test the specificity and reliability of *18-kDa M. leprae* PCR primers, RNA was extracted from PET samples of a psoriasis and an eczema biopsy material. The BI of the biopsies by acid-fast staining was negative for both samples. RNA was reverse transcribed and PCR amplification of cDNA was carried out as described earlier. Normalization of the template concentration was done using β-actin amplification (Figure 3, lanes 2–4). PCR amplification of the expected amplicon size was observed in the positive control (LL cDNA; Figure 3, lane 5). However, no amplification was observed from the eczema biopsy material (Figure 3, lane 6) and psoriasis biopsy material (Figure 3, lane 7). The data confirm that the primers used in this study are specific for *M. leprae*, *18-kDa* HSP.

M. leprae 18-kDa antigen gene is polymorphic at a single site

The *18-kDa* antigen gene was amplified from 25 leprosy samples from across the leprosy spectrum as well as reaction cases. The amplicons were purified as described earlier, and the nucleotide sequences were determined in an automated sequencer. Both the strands of the amplicons were sequenced using the *18-kDa* forward and reverse primers. Comparison of the 26 sequences with the *18-kDa HSP* gene sequence obtained from GenBank (Acc. No. M19058), showed that the 154th bp position was either T or C. Figure 4 shows a representative set of data

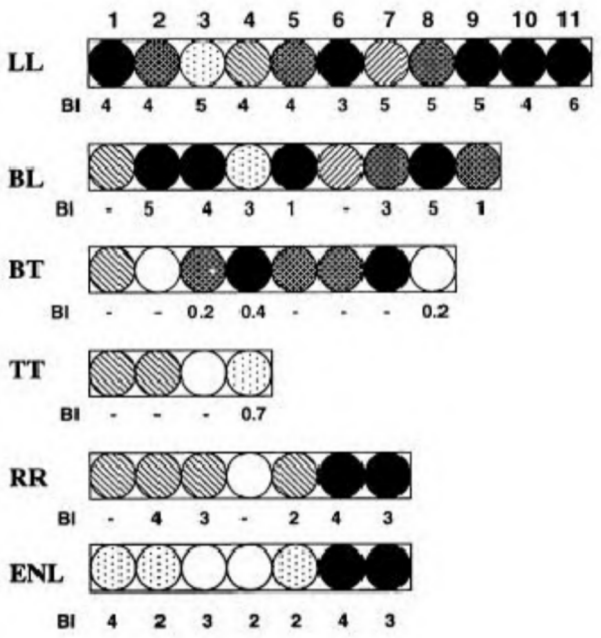

Figure 1. Semi-quantitative RT–PCR analysis of *18-kDa* antigen encoding gene in different leprosy cases. Shaded circles indicate the amplification cycles after which amplicons were visualized by UV in ethidium bromide-stained gels. Each circle represents an individual patient within the disease spectrum. The bacterial index (BI) for each patient is given below the circle.

○, Absence of PCR product after 30 cycles; ○, Presence of PCR product after 30 cycles; ◎, PCR product detectable after 26 cycles; ◉, PCR product detectable after 22 cycles; ●, PCR product detectable after 18 cycles.

for various leprosy cases. Among the PCR products analysed, 60% was found to contain T (class-I) and the rest were found to contain C (class-II) at this position (Table 2). A representative electropherogram (ABI Prism 377) of the nucleotide stretch showing the polymorphic nucleotide is given in Figure 5. The *18-kDa HSP* gene amplified from *M. leprae*-infected armadillo liver tissue was also sequenced and was found to contain T at position 154, as has been reported earlier[14]. This SNP resulted in a serine-to-proline conversion at amino acid position-52 of the 18-kDa heat shock protein (Figure 4). Interestingly, TCA to CCA change creates an additional restriction site with the sequence GATC. This could be useful for *M. leprae* strain typing.

Discussion

Comparison of whole genome sequences of isolates belonging to the same species of *M. tuberculosis*[33] and *B. anthracis*[34] indicates that genome sequence variation is more prevalent than reported. Limited genome analysis of *M. leprae* isolates indicated that the genome of *M. leprae* is highly conserved[6–9]. There are a few reports which examined the intraspecies variation in *M. leprae* and nucleotide variation of individual loci such as *RLEP*[11], *folP1*[12], *rpoB*[13]. In addition, TTC repeat polymorphism was also reported in *M. leprae*[5,35]. However, none of the above studies could reveal the true level of polymorphism in *M. leprae* isolates. In the absence of comparative whole genome sequence data for different isolates, it is essential to have as many polymorphic loci as possible for the determination of strain variation in this non-cultivable mycobacterium. In the present study, we found that the gene encoding 18-kDa heat shock protein from a population of leprosy patients from the same endemic geographical location is polymorphic at a single site.

The *M. leprae* 18-kDa HSP is a major T-cell antigen with immunodominant epitopes[29,30]. It has been reported that the T-cell clones specific for peptides carrying amino acids 1–38 and 41–55 were found to be cross reactive with *M. tuberculosis* complex, *M. avium* and *M. scrofulaceum* heat shock proteins, whereas T-cells specific for peptides carrying amino acids 38–50 were found to be *M. leprae* antigen-specific[30]. The amino acid change due to polymorphism from serine to proline in the 52nd amino acid falls in the peptide region (41–55 aa) of 18-kDa HSP, which has been identified as a T-cell epitope. It is of interest to know the effect of this amino acid change on T-cell reactivity, since these T-cell clones were generated for class-I 18-kDa HSP.

The 18-kDa HSP belongs to the family of α-crystallin, low molecular mass HSPs[14]. The *18-kDa* gene is reported to be highly specific for *M. leprae* and has been used as a molecular marker for *M. leprae* in several reports[23–27]. Sharma *et al.*[24] examined the biopsy material from Indian patients and found the presence of *18-kDa* antigen gene using *M. leprae* DNA as template. These authors reported 100% sensitivity in AFB-positive and 71% sensitivity with AFB-negative samples. In our study we could detect *18-kDa* antigen mRNA in 91% of AFB-positive and 75% of AFB-negative cases, indicating a close correlation. This result supports the reliability of RT–PCR analysis in gene identification as well. The absence of *18-kDa* anti-

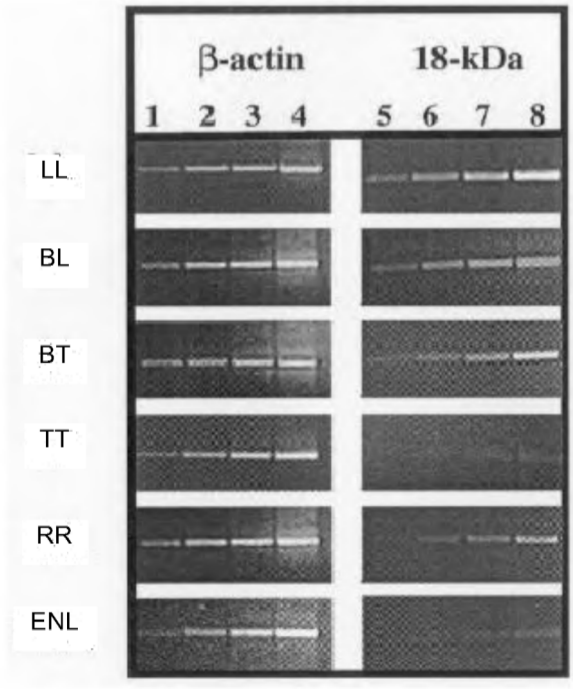

Figure 2. Semi-quantitative RT–PCR determination of human β-actin and *18-kDa HSP* gene from samples with normalized amount of cDNA templates. Lanes 1–4, Amplification of β-actin at 18, 22, 26 and 30 cycles respectively; lanes 5–8, Amplification of *M. leprae 18-kDa HSP* gene at 18, 22, 26 and 30 cycles respectively.

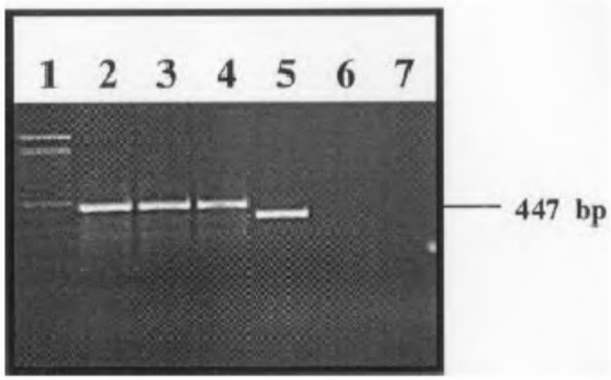

Figure 3. Absence of *M. leprae 18-kDa* antigen mRNA in non-leprosy skin conditions. Lane 1, 100 bp ladder; lane 2, β-actin (LL); lane 3, β-actin (eczema); lane 4, β-actin (psoriasis); lane 5, *18-kDa* (LL); lane 6, *18-kDa* (eczema); lane 7, *18-kDa* (psoriasis).

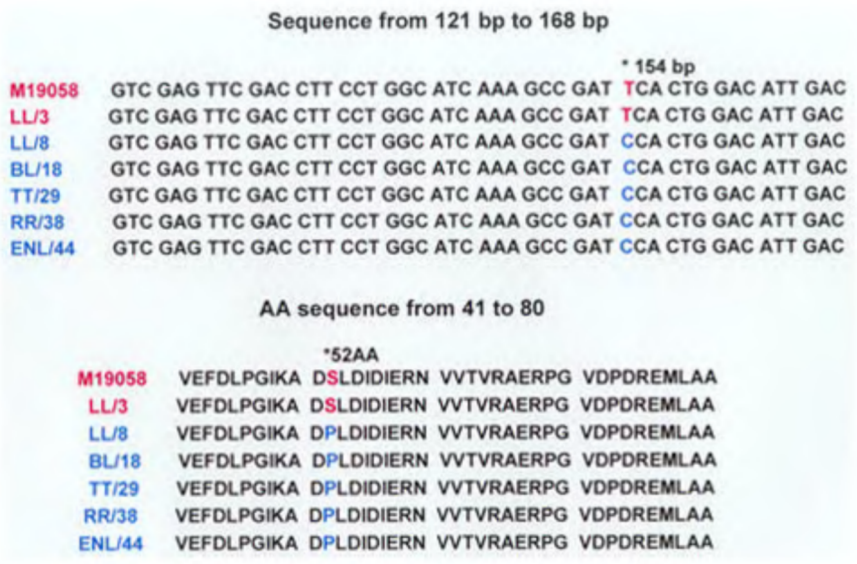

Figure 4. Nucleotide sequences of a segment of the *18-kDa* antigen gene and amino acid sequences of 18-kDa HSP peptide from different samples.

Table 2. Single nucleotide polymorphism in *18-kDa* antigen gene

Disease spectrum	Nucleotide at 154th position
LL (3, 4, 5, 6, 11)	T
LL (8, 10)	C
BL (14, 15, 19)	T
BL (18)	C
BT (24, 25, 26)	T
BT (27, 28)	C
TT (31)	T
TT (29, 30)	C
RR (37, 39)	T
RR (38)	C
ENL (40)	T
ENL (44, 45)	C

Number within brackets indicate sample number given in Table 1.

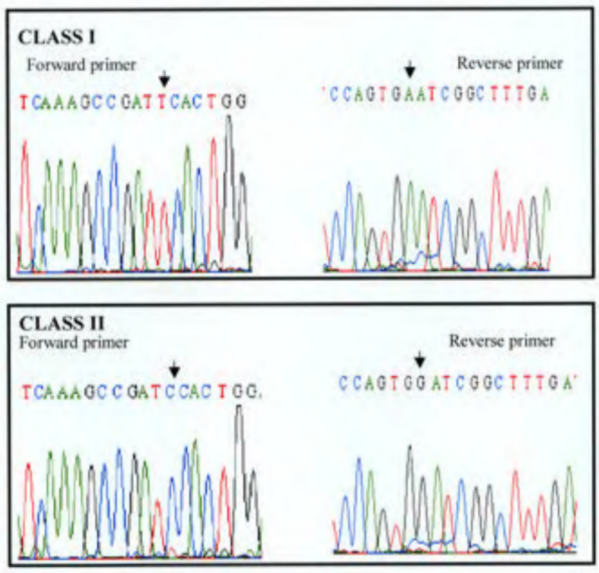

Figure 5. Representative electropherogram of *18-kDa* antigen gene. The sequence from 143 to 160 bp alone is shown for clarity.

gen-specific mRNA in eczema and psoriasis cases also shows the specificity of the primers used in this study.

Nucleotide sequence analysis revealed that a single nucleotide at 154th position exists as T or C, thereby replacing the serine codon TCA to proline codon CCA. TCA and CCA occur at about the same frequency in the 25 cases examined. The sample number is not high enough to arrive at any conclusion regarding the frequency of this mutation in various clinical forms of leprosy. However, the definitive observation is that a particular patient has either TCA or CCA containing gene, and we did not find a mixture of TCA and CCA containing genes in any of the patients so far examined. The clonal expansion of bacteria carrying only one type of *18-kDa* antigen gene indicates perhaps, the absence of secondary infection from a different strain or preferential survival of only one type of *M. leprae* strain in patients. The serial analysis of the same patient from TT to LL stage and examination of patients before and after reactional episodes will be of interest. These studies are under way.

Transcription of the *18-kDa* antigen gene indicates that metabolically viable bacteria are present in the biopsy material. The lack of correlation of mRNA levels with

BI, even in active disease condition such as LL, indicates variation in expression level. However, the treatment status of patients should be assessed before arriving at any definitive conclusion. More controlled study involving untreated cases is needed to settle this issue. In ENL cases, 5 out 7 showed the presence of *18-kDa* antigen mRNA. This opens up the possibility to examine two issues, viz. the viability of the pathogen in ENL cases and also the question of whether ENL cases have reinfection or relapse of existing bacteria. Further, it is likely that bacterial gene expression might play a crucial role in reaction condition as well. The *18-kDa* antigen gene being a macrophage-inducible gene of *M. leprae*[36], examination of the *M. leprae* profiles of this gene and other similar pathogenesis-specific genes is important to understand leprosy.

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