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Original Article

Genotypic analysis of *Mycobacterium leprae* strains from different regions of India on the basis of *rpoT*

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Mycobacterium leprae strains from Indian leprosy patients were analyzed using the six base tandem repeat, GACATC, in *rpoT* gene as genetic marker. DNA was extracted from slit-skin smears and nasal swabs of new untreated as well as treated leprosy patients living in different regions of India. PCR amplification of *rpoT* gene and sequencing of amplicons showed the presence of two genotype of *M. leprae* in this study, 73.4% having three copies (ancient Indian type) and 26.6% contain 4 copies (considered to be Japanese and Korean). These genotypes alongwith other short tandem repeats may help in studying the historical spread of disease and the strains of *M. leprae* disseminated by various human races that migrated to India from other places of Asia and European countries during our history.

Key words : Genotypic analysis, *rpoT*, *Mycobacterium leprae*

Introduction

Leprosy is a disease of great antiquity having been recognized from Vedic times in India and from Biblical times in the Middle East. Leprosy may be defined as a chronic, infective disease of low contagiousness that affects primarily the superficial parts of the body especially nerves and appendages of the skin like sweat and sebaceous glands in susceptible hosts after a varying incubation period. *Mycobacterium leprae* was identified as a cause of leprosy in 1873 by Norwegian physician Dr Gerhard Henrik Armauer Hansen.

Leprosy is still a health problem globally and a total of 1.38 lakh new cases were detected in India during the year 2007-08 which gives annual new case detection rate (ANCDR) of 11.70 per 100,000 population (NLEP 2008). In order to understand the high occurrence of leprosy in some areas, it is necessary to identify the natural reservoir(s) of *M. leprae*, the route of infection and the mode of transmission. The exact mode of transmission is not fully known and the possible routes under consideration are the skin and the respiratory tract. There are more and more evidences that leprosy may be transmitted via aerosols containing *M. leprae* (droplet infection). With the

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realization of the importance of the nose as a portal of exit, there has been increased emphasis on the respiratory tract as the portal of entry. In addition, numerous studies indicate that leprosy is transmitted from person-to-person by close contact between an infectious patient and a healthy but susceptible host. The actual importance of transmission channels is not known.

Several rapid molecular assays have been developed for detection of *M. leprae* directly from patient specimens and these assays have been primarily based on the amplification of *M. leprae* specific sequences using PCR and identification of *M. leprae* DNA fragment (Cole et al 2001). In addition, different genotyping methods have been/ are being developed. These molecular biological techniques can help in devising techniques for understanding the epidemiology of leprosy and identifying sources as well as causes of persisting foci of disease. Molecular methods to elicit strain differences within the leprosy bacillus would be especially important for this purpose. Molecular typing will make it feasible not only to study the global and geographical distribution of distinct clones of *M. leprae* but also to correlate between the *M. leprae* and the type of disease manifestation and provide some insight into historical and phylogenetic evolution of the bacillus that has affected humans and stigmatized leprosy patients for centuries (Monot et al 2005). A number of genes have been reported as targets for eliciting polymorphism in *M. leprae.*

With the publication of complete genome sequence of *M. leprae*, there is opportunity to identify novel targets to elicit genetic differences. Williams et al (1990) reported that *M. leprae* isolates obtained from geographically distinct areas did not exhibit significant genotypic diversity by RFLP. On the other hand, Katoch et al (1998) observed some variation in this region in their findings. Some of the promising targets for eliciting strain differences among *M. leprae* are TTC (Shin et al 2000), *rpoT* (Matsuoka et al 2000, 2005), VNTRs (Groathouse et al 2004, Truman et al 2004) and SNPs (Monot et al 2005, Matsuoka et al 2006). As reported by Matsuoka et al (2000, 2005), *rpoT* gene has a hexanucleotide repeat and has been found to be present in three copies in most strains of *M. leprae* including TN Indian strain but in four copies in eastern Asian countries such as mainland Japan and Korea. An earlier study from north India reported predominance of strains with 3 copies (89%) against 11% with 4 copies (Lavania et al 2007).

This study focuses on determination of the genotypes of *M. leprae* from the leprosy patients from different regions of India based on polymorphism in *rpoT* gene.

Materials and Methods

Source of *M. leprae* strains

One hundred twenty slit-skin scrapings (SSS) in 70% ethanol and forty six nasal swabs from multibacillary as well as paucibacillary leprosy patients were collected in Tris-EDTA buffer from the Out Patient Door of The Leprosy Mission (TLM) Hospitals at Shahadara, Delhi (69 SSS), Purulia, West Bengal (28 SSS and 46 swab) and Miraj, Maharashtra (23 SSS), India. Samples were stored at -20°C until DNA extraction. All the experiments were performed in Stanley Browne Laboratory, TLM Community Hospital, Nand Nagari Delhi.

Extraction of genomic DNA

M. leprae DNA was isolated from slit-skin smear by following overnight lysis with proteinase K. The reaction was terminated at 97°C for 15 minutes. This lysate preparation was further used for PCR. DNA from nasal swabs was extracted as described earlier (Jadhav et al 2002).

Genotyping of *rpoT* by PCR and electrophoresis

PCR amplification was carried out as per published procedure (Matsuoka et al 2000). A total 25 µl of reaction volume contained 3 µl of template DNA, 1mM of each primer at final concentration of 0.5 µM (forward and reverse) and 1x Genei Mix.

Primers A (5'-ATGCCGAACCGGACCTCGACGTTGA-3') and B (5'-TCGTCTTCGAGGTCGTCGAGA-3') (GenBank Accession No. AB019194) were used for amplification to span the 91bp (containing 3

repeats) or 97-bp (containing 4 repeats) fragments of the target *rpoT* gene. PCR reaction without DNA was used as negative control. Purified *M. leprae* DNA was used as positive control. The amplification was carried out in a thermal cycler (Corbett JH BIO) under the conditions of 95°C for 5 min for initial denaturation followed by 45 cycles, each cycle consisting of denaturation at 95°C for 30 sec, annealing at 60°C for 2 min and extension at 72°C for 4 min with a final extension at 72°C for 10 min. For identifying the differences in the repetitive region of the *rpoT* gene, 15 µl of each PCR product containing amplified fragment of the target region was electrophoresed along with DNA ladder of 100 base pair in a 4% low melting point agarose gel (Sigma) using Tris-Borate-EDTA buffer at 100 volts constant current. Size of amplicons were analyzed with the help of DNA ladder using the software Alpha Imager.

Fragment length analysis

Samples were sent to Lab India at Gurgaon, India for fragment length analysis. After PCR amplification, 1 µl of the PCR product was diluted 30- to 60-fold, and 1 µl of the diluted PCR product was combined with 12 µl deionized formamide (Applied Biosystems) and 0.3 µl of the LIZ-500 DNA standard (Applied Biosystems). The sample was denatured at 94°C for 5 min and subjected to capillary electrophoresis on the Applied Biosystems genetic analyzer. The electropherograms were visualized and analyzed using GeneMapper.

Ethical approval

Informed consent was obtained from all the patients and the study was approved by the Institutional Ethical Committee (IEC).

Results

We could amplify fragment of *rpoT* gene from 139 clinical samples out of 166 samples (Table 1). DNA from 95 slit-skin scrapings and 44 nasal swabs samples showed amplification. PCR amplicons of different sizes, 91 bp or 97 bp of *rpoT* gene were obtained (Figure 1 and Table 1). We observed that there was complete concordance between sequencing and gel electrophoresis results. Out of 139 samples, 102 (73.4%) samples showed 91 bp amplicon (three copies of repeats) whereas 37 (26.6%) showed 97 bp amplicon (four copies). Among *M. leprae* isolated from leprosy patients (51) from northern India (Shahdara, Delhi), 84.3% strains exhibited a band of 91 bp corresponding to three copies of the six base pair tandem repeat while remaining 15.7% strains showed 97 bp band corresponding to four copies of the repeat.

We looked at pattern only in slit-skin smear samples (Table 2). Total 120 slit-skin smear samples were tested of which we could get amplification for fragment of *rpoT* gene from 95 samples. All the slit-skin smear samples that failed to give amplification were from BI negative MB or PB cases. As described before majority of the samples had three repeats. Difference that we see between the samples from north India (Delhi) and other parts of India (western and eastern part of India) was examined for any statistical significance using *chi*-square test. The difference appears to be statistically significant ($p<0.05$). This was compared with pattern in nasal swabs. Among the 44 nasal swabs samples, 30 (68.2%) exhibited 3 copy and 14 (31.8%) were exhibiting 4 copy number of hexanucleotide repeat in *rpoT* gene. Among 95 slit smear scrapings, 70 (75.8%) were showing 3 copy number while 23 (24.2%)

Table 1 : Distribution of two genotypes in different areas

City/ State	Three repeats	Four repeats	Negative	Total samples
Purulia	46 (66.7%)	23 (33.3%)	5	69 (74)
Miraj	13 (68.4%)	06 (31.6%)	4	19 (23)
Shahdara	43 (84.3%)	08 (15.7%)	18	51 (69)
Total	**102 (73.4%)**	**37 (26.6%)**		

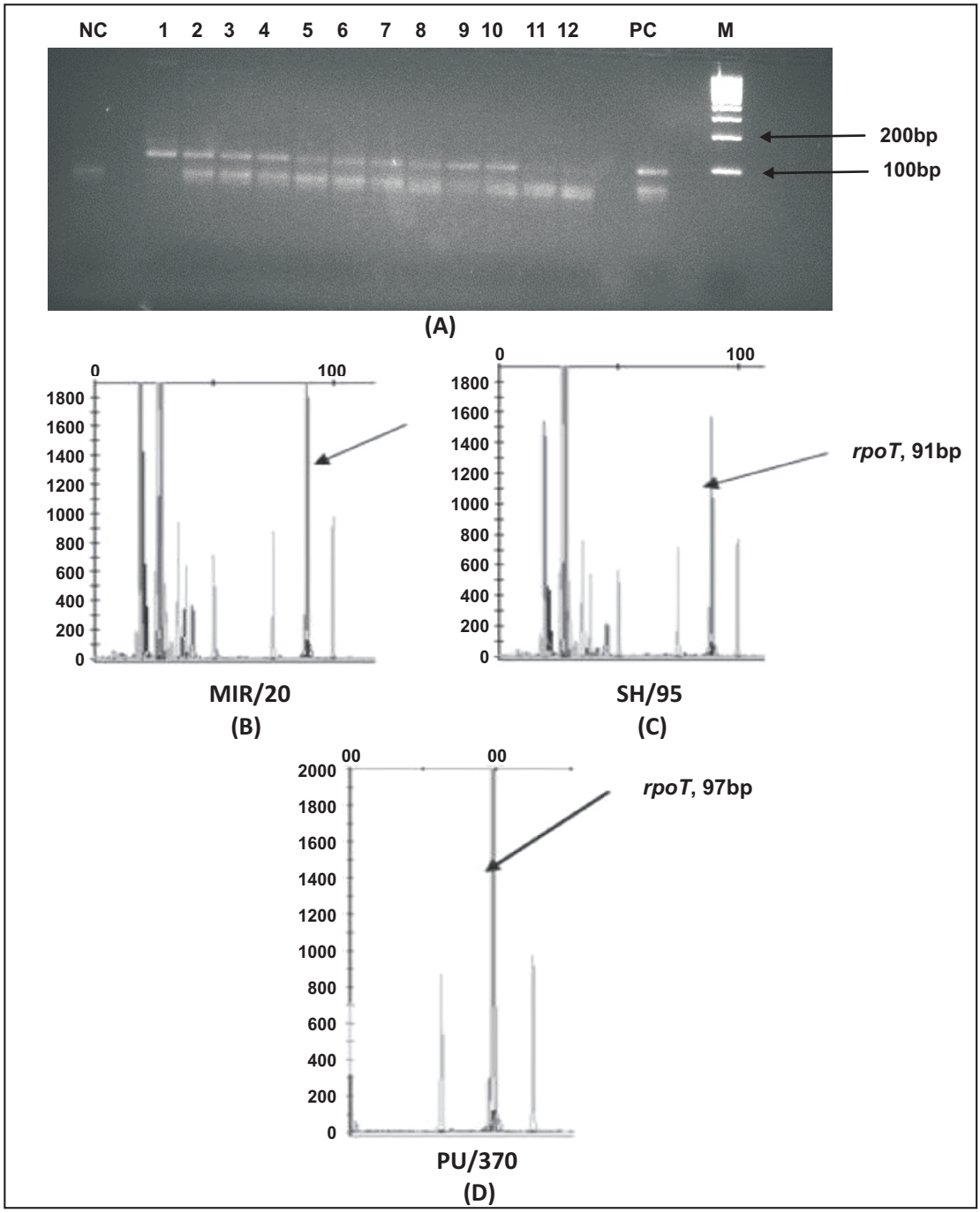

Figure 1 : (A) Gel electropherograms of *rpoT* amplicons from patients from different regions (Lane 1-12 : Samples, PC : Positive control, NC : Negative control, M : Molecular marker) (B) MIR/20 : Patients from Miraj (C) SH/95 : Patients from Shahadara and (D) PU/370 : Patients from Purulia

Table 2 : Results of *rpoT* for slit-skin smears

City/ State	Three repeats	Four repeats	Negative	Total samples
Purulia	16 (64%)	9 (36%)	0	25 (25)
Miraj	13 (68.4%)	6 (31.6%)	4	19 (23)
Shahdara	43 (84.3%)	8 (15.7%)	18	51 (69)
Total	**72 (75.8%)**	**23 (24.2%)**		

Table 3 : *rpoT* results from slit-skin smears and nasal swabs

Source	Three repeats	Four repeats	Negative	Total samples
Slit-skin smear	72 (75.8%)	23 (24.2%)	25	95 (120)
Nasal swab	30 (68.2%)	14 (31.8%)	2	44 (46)
Total	**102 (73.4%)**	**37 (26.6%)**		

were having 4 copy number of repeats. The higher percentage of 3 copy number in slit-skin smear samples compared to nasal swabs (though statistically not significant) was due to higher percentage observed in samples from Delhi. When we checked for slit-skin smear samples only from Purulia and Miraj, we observed 29 out of 44 slit-skin smear samples showing 3 copy numbers whereas among 44 nasal swab samples, 30 samples showed three copy number which was almost same (Table 3).

Discussion

In the present study, the patients were from far flung areas of India belonging to different areas such as Delhi (north India), Purulia (eastern part of India) and Miraj (western part of India). *M. leprae* containing the four copy of the repeat was relatively more in the Purulia district (33% among all other states) of West Bengal and Miraj (31.6%) compared to Delhi (15.7%). However, the sample size from Miraj is small to draw any conclusion.

Little is known about Purulia before the East-India Company obtained the 'Diwani' of Bengal, Bihar and Orissa in 1765 (http://en.wikipedia.org/wiki/Purulia_district viewed as on 13th August 2009). Kolkata (Calcutta) served as the capital of India during the British Raj until 1911. Foreign trade was carried on by land and sea. Merchants sailed overseas from port cities. Purulia has geographical significance because of its tropical location and its shape. It functions like a funnel not only for the tropical monsoon current from Bay to the subtropical parts of north-west India but also acts as a gateway between the developed industrial belts of West Bengal and the hinterlands in Orissa, Jharkhand, Madhya Pradesh and Uttar Pradesh. Miraj is a part of Sangli district which was a princely state and was ruled by Chalukyas in 12th century and later on was a part of Maratha Empire. This place is also one of the major trading centre for turmeric in Asia (http://en.wikipedia.org/wiki/Sangli as viewed on 13th August 2009).The Indian capital city of Delhi has a long history, including a history as the capital of several empires and British period (http://en.wikipedia.org/wiki/History_of_Delhi as viewed on 13th August 2009).

It has been reported that the prominent biased geographical distribution of 4 copy type in Korea and main island of Japan was attributed to historical movement of mongoloid in this area (Matsuoka et al 2000). Our study also depicts several characteristic features of *M. leprae* from different regions of India. This 26.6% sub-population of *M. leprae* in our samples with four copies of the hexanucleotide repeats in *rpoT* gene might have originated from migrants from the different countries in the past as described in the history. In a previous study (Lavania et al 2007),

11% of the north Indian strains were exhibiting 4 copy numbers and our findings support that data. In another study from Latin America, Matsuoka et al (2005) reported that genotypes with four repeats in the *rpoT* gene was predominant in Mexico but the genotype from Peru and Paraguay contained three repeats in the *rpoT* gene. By comparing single nucleotide polymorphisms (SNPs), analysis of polymorphism in seven variable number of tandem repeat (VNTR) and *rpoT* gene in the strains of *M. leprae* from Japan and other Asian countries, Monot et al (2005) concluded that leprosy was introduced into west Africa by infected explorers, traders or colonialists of Europeans or north African descent.

It is believed that one or two unit differences in copy number are expected in loci prone to high rates of mutation due to the long incubation period of the disease and unknown number of transmission cycles before the onset of clinical disease. However, more such studies are needed and in larger group of patients to study the strain diversity of *M. leprae.* The present study was done on the slit-skin scrapings of leprosy patient from our field unit. In our study, we observed a prominent biased distribution of *M.leprae* with dominant three copies of the six base tandem repeats in the *rpoT* gene in India. Alongwith these findings, our data also have suggested a clear promise of use of slit-skin scrapings and nasal swabs for molecular typing to study the transmission of leprosy which will be very important in understanding epidemiology of the disease and moving towards ultimate eradication of the disease.

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