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Strain Typing and Strain Differentiation of *Mycobacterium leprae* by TTC Repeats

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Abstract: The main pathogen based molecular markers involved in strain typing and strain differentiation are SNP and VNTR. A typing system for global differentiation of *M. leprae* is the analysis of variable number tandem repeats based on STRs. To expand the analysis of *M. leprae* TTC gene polymorphism, this study involved strain typing of 14 Lepromatous Leprosy (LL) SSS samples. For the strain typing and strain differentiation of *M. Lprae*, the present study involves 14 bacteriological positive Lepromatous Leprosy (LL) Slit Skin Smear (SSS) samples. Most of cases were identified as male and 13 cases have associated with nerve involvement. Two cases with tenderness and two with eye involvement also have been screened. DNA was extracted from SSS of 14 untreated LL leprosy cases. PCR amplification of TTC gene and sequencing of amplicons showed the allelic variation and percentage of distribution of allele in the enrolled samples. Among the 14 SSS samples, 1 (7%) exhibited 9 copy number, 1 (7%) associated with 10 copy number, 6 (43%) identified as 12 copy number, 1 (7%) known as 13 copy number, 1 (7%) exhibited 15 copy number, 1 (7%) linked with 16 copy number, 1 (7%) exhibited 18 copy number and 2 (15%) identified as 19 copy number of (TTC) 21 allele. However, the positive strain called TN was associated with standard repeat units of TTC that is 21. As conclusion we found that, the present results has demonstrated that TTC repeats of leprosy patients has 12 and 19 copy number as a abundant which was slight differ from other leprosy strains from India.

Key words: *Mycobacterium leprae*, TTC repeats, variable number tandem repeats, strain typing

INTRODUCTION

Leprosy is chronic infectious disease, which is associated with the disability, stigma and discrimination to the affected individuals. Multi Drug Therapy (MDT) is the main source to treat the leprosy individuals as well as to diminish the transmission of leprosy (Rinaldi, 2005). According to the WHO reports, over last 15 years the leprosy is rapidly decline but the newly registered cases found in the year 2011 is 219000 which suggests that the transmission of leprosy still occur in the world, especially in the regions of endemic. In such areas the strain typing and strain differentiation are very helpful to identify the source of infection, transmission of infection, spreading of disease, differentiating cases of relapse from reinfection and for unraveling possible links between human and non reservoir sources (Linder *et al.*, 2008; Shetty *et al.*, 2005).

The main methods involved in the strain typing leprosy are RFLP, SNP and VNTR. RFLP is successfully

useful for detection of low amount *M. leprae* DNA, so that it considered as rapid, sensitive and specific test. Previous reports explain that RFLP studies of *M. leprae* were allied with lower genetic diversity. For identification of long range transmission leprosy, SNP studies are very useful provided these SNPs can be specific for particular strain and are normally uninformative but shared by 2 or more related strains where they provide as informative markers. So, far 4 SNP types were identified and these are further classified into 16 sub types designated as SNP1A-D, SNP2F-H, SNP3I-M and SNP4N-P. The genetic diversity of *M. leprae* is very low which was successfully typed basing on Short Tandem Repeats (STR) or microsatellites (repeat length 2-5 bp) and minisatellite (repeat length 6-50 bp) called as VNTR (Keim *et al.*, 2000).

Based upon the VNTR marker, several studies were conducted to see the broad range allelic diversity in the strains of leprosy and from that allelic diversity strain typing, strain differentiation and transmission of leprosy

were carried out in the world from epidemiological level (Cardona-Castro *et al.*, 2009; Fontes *et al.*, 2009; Kimura *et al.*, 2009; Sakamuri *et al.*, 2009a; 2009b; Srisungnam *et al.*, 2009; Weng *et al.*, 2007). Some of the studies on strain variations of leprosy have been carried out in India (Shinde *et al.*, 2009; Kuruwa *et al.*, 2012). Recently several strain typing methods were performed with single loci. The variations in STR with 6-bp repeat of *rpoT* were identified as associated with global transmission of leprosy (Matsuoka *et al.*, 2000; Zhang *et al.*, 2005; Matsuoka *et al.*, 2006). Same studies were performed predominantly in Japan and Korea and recognized as two copy type, 3-copy type and 4-copy type of *rpoT*. Besides these studies, several other were also eliciting in India for strain differences among TTC and *rpoT* (Matsuoka *et al.*, 2006; Shin *et al.*, 2000). In the present study we elucidated strain variation and strain typing through polymorphism TTC repeats of 14 Lepromatous leprosy SSS DNA samples.

MATERIALS AND METHODS

Sample collection: LEPROA India is a nongovernmental organization which takes care for patients in Andhra Pradesh, Assam, Bihar, Jharkhand, Madhya Pradesh, Odisha and Sikkim states of India. As referral centre, it connected with the patients in free diagnosis, free treatment of leprosy, TB, HIV, fileriasis and eye care. The Blue Peter Public Health and Research Centre (BPHRC) is research wing of LEPROA-India which attain samples from the entire referral centers of India for the research purpose. So that in this study we selected, 20 LL patients from Andhra Pradesh and Orissa for VNTR study. The clinical finding, other relevant history was done by medical officer or physician and information was summarized in Table 1. From suspect leprosy patients, the SSS (Slit Skin Smear) were obtained with informed consent

and normally sample collected from left ear lobe, right ear lobe and fore head with separate scalpel blade. Skin scrapings from each site were stored in a separate aliquot containing 70% ethanol and then stored at -20 until DNA extraction was done.

DNA extraction: The total DNA from ethanol fixed SSS were extracted through DNeasy Blood and Tissue Kit, Qiagen, Germany (cat No.69504) according the manufactures instructions, briefly ethanol fixed sample was neutralized with phosphate buffer followed by ATL and proteinase-K treatment. Break down of cell wall and cell membrane was carried out by overnight incubation of sample at 55°C then the sample was mixed with AL buffer and ethanol and heat at 70°C for 20 min. Transfer the sample into spin columns, washed the sample with AW1 and AW2 buffer. Finally, the DNA was eluted in 50-100 µL with AE buffer. The DNA was stored in -4°C until PCR was performed.

Amplification of TTC repeats: For the amplification of TTC repeats, the primers were selected from earlier reports (Shin *et al.*, 2000). These selected primers were designated as TTC-F 5'GGACCTAAACCATCCCGTTT-3' and TTC-R 5'CTACAGGGGGCACTTAGCTC-3' ordered from Eurofins Genomics India Pvt. Ltd, Bangalore. The ingredients used and PCR cycling conditions were also obtained from previous reports (Sakamuri *et al.*, 2009b) briefly, 25 µL of reaction mixture contains 12.5 µL of red dye master mix, 8.5 µL of nuclease free water, 1 µL forward primer, 1 µL reverse primer and 2 µL of genomic DNA. The simple PCR conditions included, initial denaturation at 95°C for 10 min; 94°C for 90 sec, 60°C for 90 sec and 72°C for 90 sec min for 40 cycles; final extension at 72°C for 10 min; termination and storage of PCR samples were kept at 4°C. 2% agarose was used to perceive the positive amplification of TTC repeats.

Table 1: General information and clinical classification of the patients including Bacteriological Index (BI)

| PHC code | Patient ID | Age (years) | Sex | Marital status | No. of lesions | Nerves involved | Tenderness | Deformity | Eye involvement | Clinical classification | Ridley-Jopling | BI |
|----------|------------|-------------|-----|----------------|----------------|-----------------|------------|-----------|-----------------|-------------------------|----------------|-----|
| AD | 12/12 | 35 | M | Married | >5 | Yes | Yes | No | No | MB | LL | 4+ |
| H1 | 007/12 | 36 | M | Married | >5 | Yes | Yes | No | No | MB | LL | 3+ |
| H1 | 114/12 | 37 | M | Married | >5 | Yes | No | No | No | MB | LL | 6+ |
| H1 | 085/12 | 23 | M | Married | >5 | Yes | No | No | No | MB | LL | 5+ |
| H1 | 007/13 | 27 | M | Married | >5 | Yes | No | No | No | MB | LL | 2+ |
| H1 | 014/13 | 22 | M | Single | >5 | Yes | No | No | No | MB | LL | 5+ |
| H1 | 027/12 | 20 | M | Single | >5 | Yes | No | Yes | No | MB | LL | 3+ |
| H1 | 021/13 | 45 | M | Married | >5 | Yes | No | No | No | MB | LL | 5+ |
| S1 | 003/12 | 75 | M | Married | >5 | Yes | No | Yes | Yes | MB | LL | 4+ |
| S1 | 027/12 | 48 | F | Widowed | >5 | Yes | No | No | No | MB | LL | 2+ |
| S1 | 025/12 | 60 | M | Single | >5 | No | No | No | No | MB | LL | 2+ |
| K1 | 02/2013 | 45 | M | Widowed | >5 | Yes | No | No | No | MB | LL | 4+ |
| K3 | 52/2012 | 30 | F | Married | >5 | Yes | No | No | No | MB | LL | 1+ |
| K4 | 02/2013 | 60 | M | Married | >5 | Yes | Yes | No | Yes | MB | LL | 0.5 |

M: Male, F: Female, MB: Multi bacillary, LL: Lepromatous leprosy and BI: Bacterial index

Sequencing analysis: The numbers of TTC repeats were confirmed by direct sequencing. The positive amplified products were first subjected to purification by DNA purification kit according to the manufactures instructions. PCR products with positive amplification signals were subjected for sequencing analysis at Vinta Labs Pvt. Ltd, Hyderabad, India. Sequences were generated on ABI genetic Analyzer 3730 (Applied Biosystems) and a nucleotide BLAST of the sequence of samples was performed with *M. leprae* genome at LEPROMA for detection of mutation, if any. The repeated units of TTC were read by counting of repeat units in the TTC allele gene sequence.

RESULTS

SSC were obtained from a total of 14 LL leprosy cases after clinical manifestation and other relevant history was done and their patient history was summarized. All cases were MB cases with good bacterial load. The TN strain were taken as reference to see the sensitivity, specificity and reproducibility of TTC. The primers used for the amplification of single TTC region of 14 SSS samples, were successfully amplified and were detected in 2% agarose gel, the amplified product was identified as 200 bp and it showed good concordance between gel electrophoresis and sequencing. The red dye master mix is solution contain all ingredients for PCR and it optimize the all the problems associated amplification.

We looked at pattern of TTC repeats only in slit-skin smear samples. A total of 14 highly bacterial positive Lepromatous Leprosy (LL) samples have been performed to simple PCR by using specific primers. As a high bacterial positivity, all the samples were associated with good QC output. Gel checking of positive control and representative samples were done with 2% gel and were summarized in Fig. 1. The TN strain was taken as reference to see the sensitivity, specificity and reproducibility of TTC by identifying repeating units of TTC and electropherogram and complete gene sequence of TN strain was addressed in Fig. 2 and 3, respectively. We observed that there was complete concordance between sequencing and gel electrophoresis results. For analysis of VNTR of TTC locus, the products were done to sequencing in applied biosystems and resulted were obtained by blasting of sequence in NCBI nucleotide blast. Based upon the referral sequence, TTC repeat unit variation of samples was identified. Electropherogram and complete gene sequence of representative samples with TTC discriminatory were addressed in Fig. 4 and 5, respectively.

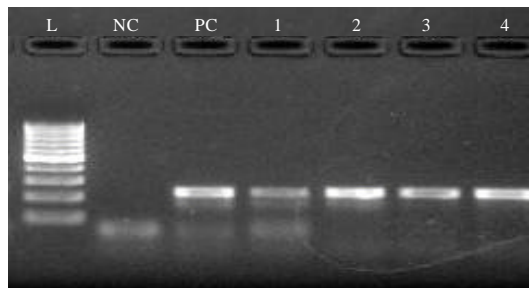


Fig. 1: Amplification products of TTC repeats produced on 2% agarose gel, L: Ladder=Blank=Negative control=Positive control and 1, 2,3,4 = Different samples

Total 14 slit-skin smear samples were tested of which we could get amplification for fragment of TTC gene in all the samples. The percentage distribution of TTC allele was summarized in Fig. 6. Among the 14 SSS samples, 1 (7%) exhibited 9 copy number, 1 (7%) associated with 10 copy number, 6 (43%) identified as 12 copy number, 1 (7%) known as 13 copy number, 1 (7%) exhibited 15 copy number, 1 (7%) linked with 16 copy number, 1 (7%) exhibited 18 copy number and 2 (15%) identified as 19 copy number of (TTC) 21 allele. However, the positive strain called TN was associated with standard repeat units of TTC that is 21.

DISCUSSION

Sequence changes in DNA is the main approach to carry out molecular based typing which can arise from single nucleotide changes, deletions and insertions (such as those arising from mobile elements) and repetitive stretches of sequences (tandem repetitions) at different sites of a genome. The variation in repeat units in DNA is excellent source of polymorphism and it occurs due to errors in replication by polymerase enzyme. The most recent advance has been in the molecular typing of the VNTRs by PCR amplification, multiplex electrophoresis and automated detection and analyses (Supply *et al.*, 2001). The understandings of strain typing and strain differentiation of *M. leprae* are very essential to see the surveillance and prevent an infection. The earlier findings on seroepidemiology indicated widespread *M. leprae* infections within a population (Abe *et al.*, 1990; Cho *et al.*, 1992; Izumi *et al.*, 1999; Van Beers *et al.*, 1994) and studies by PCR on the distribution of the bacilli also found that many individuals in areas in which leprosy is endemic. The present study



Fig. 2: Electropherogram of positive control, TTC repeats were with different colours

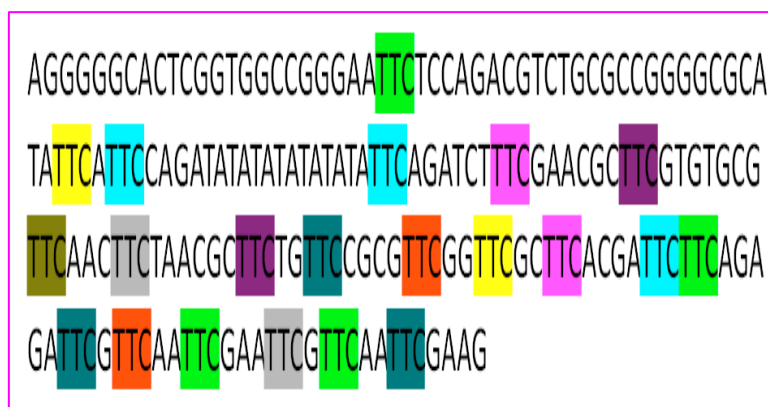


Fig. 3: Complete genome sequence of positive control, TTC repeats were with different colours

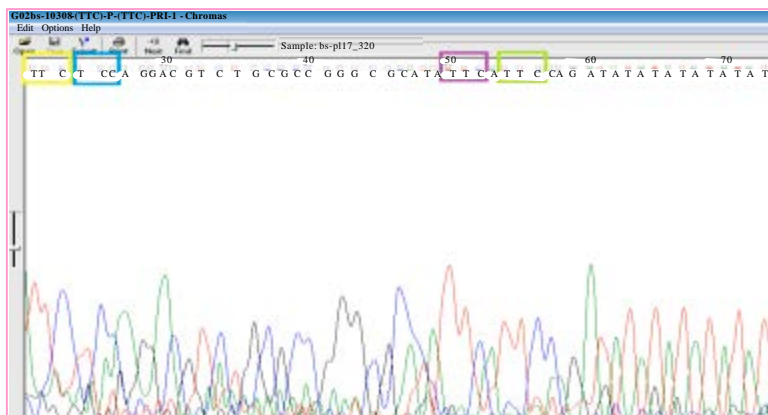


Fig. 4: Electropherogram of representative sample, TTC repeats were with different colours

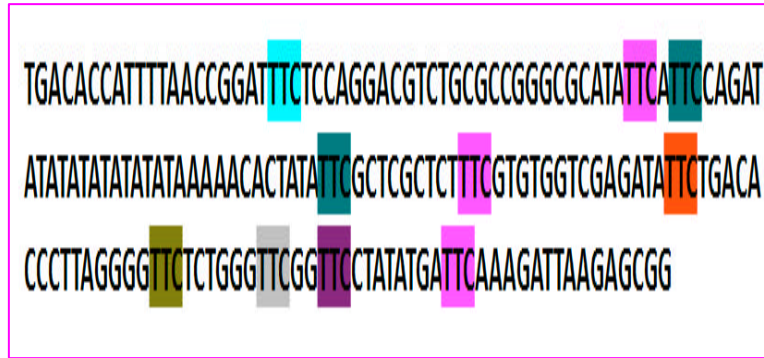


Fig. 5: Complete genome sequence of representative sample, TTC repeats were with different colours

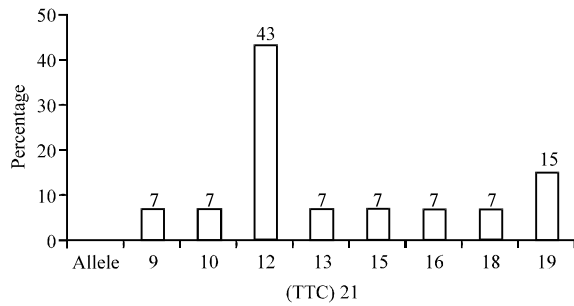


Fig. 6: Bar graphs showing percent distribution of (TTC) 21 repeats

was to look for strain typing and strain differentiation of mycobacterium leprae from selected affected leprosy individuals.

Several mycobacterium species like *Mycobacterium tuberculosis*, *Mycobacterium marinum*, *Mycobacterium avium* were not contain the TTC repeat allele gene which specify that *Mycobacterium leprae* is the only organism having TTC repeat in Mycobacterium species. *Mycobacterium leprae* was tested for the stability TTC repeat gene form the tissues of armadillo and nude mice and this investigation was done for 121 months, so this gene is found to be good marker to look strain differentiation for epidemiological investigations of leprosy. Several studies were reported to evaluate strain differentiation based on single allele. Strain typing based upon rpoT in Indian leprosy patients was clear estimation of strain variation in mycobacterium leprae (Lavania *et al.*, 2009). The promising targets for eliciting strain differences among *M. leprae* by TTC was good supportive to strain typing (Shin *et al.*, 2000). In the present study we evaluated the strain typing and strain differentiation of some of the south Indian patients by

Variable Number Tandem Repeats (VNTR). The study was conducted by taking selected patients from BPHRC clinic Hyderabad, India as it is government recognised referral centre of leprosy. Sample was collected from suspected leprosy individuals by informed consent and clinical manifestation was done by medical officer. The sample was processed and DNA was further subjected VNTR analysis under universal conditions to see the amplification of TTC loci. Most of the VNTR and sequence results were associated with high positive BI Multi Bacilli samples due to good concentration of DNA. The samples having low BI showed poor FLA results which further repeated.

The (TTC) 21 repeats of locus (TTC) 21 locus were addressed taking strains from Thai-53, Kyoto-1, Zensho-4 and Korea 3-2 and 17 samples, namely, the fourth generation of Thai-53 (Thai-53 4th), Thai-53 7th, Thai-53 11th, Kyoto-1 3rd, Kyoto-1 5th, Kyoto-1 7th, Kyoto-1 8th, Zensho-4 (biopsy specimen), Zensho-4 1st, Zensho-4 2nd, Zensho-4 3rd, Zensho-4 4th, Korea 3-2 (biopsy specimen), Korea 3-2 1st, Korea 3-2 2nd, Korea 3-2 3rd and Korea 3-2 4th for allelic variation of (TTC)21 locus and these results demonstrated that these strains contain 9 to 25 copy number and 11 copy number was identified as predominant a predominant (Zhang *et al.*, 2005). Specimens were from patients attending the Leonard Wood Memorial Skin Clinic in Cebu, the Philippines, collected in collaboration with Yonsei University have been investigated to allelic variation of TTC locus and these results demonstrated 10 to 28 copy number was common and 13 and 10 copy number was high (Kimura *et al.*, 2009).

In south Indian leprosy patients 18 and 15 copy number of (TTC) 21 have predominant (Shinde *et al.*, 2009) but in the present study 12 copy number was high. Maharashtra leprosy patients have

been identified as 12 number as high which alike of the present study (Kuruwa *et al.*, 2012). Our present results from Indian patients were clearly indicated that TTC is a conserved repeat region and stable in all Indian leprosy patients. Genomic polymorphism of *M. leprae* by Variable Number Tandem Repeats were successfully discriminates the from Indian leprosy patients. The present study was mainly based on strain typing of *Mycobacterium leprae* by Variable Number Tandem Repeats using specific prospective study design from 14 LL leprosy affected individuals. Most of the samples showed 12 (43%) repeats of TTC followed by 19 (15%) times. Rest of the samples have described as low repeat number. The present results has demonstrated that TTC repeats of leprosy patients has 12 and 19 copy number as a abundant which was slight differ from other leprosy strains from India.

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