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Research Article

MOLECULAR TYPING OF *MYCOBACTERIUM LEPRAE* BY (TTC)21 REPEATS IN MULTICASE FAMILIES

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ABSTRACT
A typing system for global differentiation of *M. leprae* is the analysis of variable number tandem repeats based on Short Tandem Repeats (STR). To expand the analysis of *M. leprae* (TTC)21 gene polymorphism and transmission of leprosy bacilli, this study involved strain typing of families of 8 active leprosy cases and their households 14 Leprosy cases. Overall 23 leprosy cases were strain typed by (TTC) 21 to see the allelic variation and strain transmission. The strain typing results revealed that, 12 and 10 copy number of (TTC) 21 locus was predominant over these leprosy cases. Typing of families of leprosy cases and their household leprosy cases have been demonstrated that 4 families and their households possess same copy number of (TTC) 21 repeats, suggested households get infected with leprosy cases of family. Rest of 4 families and their households strain typing stated that these patients have different copy number of (TTC) 21 repeats, suggested that household get infected with leprosy bacilli from sounding areas not from leprosy cases of family.

Keywords: *Mycobacterium leprae, (TTC) 21 Repeats, Variable Number Tandem Repeats, Molecular Typing, VNTR and Multicase Families*

INTRODUCTION
Mycobacterium leprae is oldest pathogen which is causative agent leprosy and associated with disability in individuals (Scollard *et al.,* 2006). The proper Multi Drug Therapy (MDT) is the only treatment to prevent the disease and consequently disrupt further transmission disease to others. Although using of MDT in correct way, the declining of leprosy cases neither were drastically observed with 232, 857 cases reported cases globally in 2012 (WHO, 2012). This new case enrolment suggested that leprosy bacilli still associated with the transmission of leprosy in endemic areas. However molecular typing method helps mode of transmission thereby to aid prevents new infection of leprosy. The genome wise validation of *M.leprae* were essential realize they actually cause and transmission of the diseases.

In recent years molecular typing methodologies have complemented conventional infectious disease. With the publication in 2001 of the complete genome sequence of an isolate from Tamil Naidu, India called TN strain; selection of potential polymorphic genomic markers for strain typing was feasible. This contained short tandem repeats (STR) regions that had potential for genetic polymorphism by expansion or contraction of repeats and therefore for strain typing of *M. leprae*. A list of loci were targeted for strain typing (Shin *et al.,* 2000; Matsuoka *et al.,* 2000) and multi locus variable number tandem repeat analysis (MLVA) as means for molecular differentiation of *M. leprae*, within and amongst leprosy patients emerged (Groathouse *et al.,* 2004). Globally several studies using one or more loci have been published for isolates from Colombia, Brazil, Philippines, Thailand and China (Cardona *et al.,* 2009; Fontes *et al.,* 2009; Sakamuri *et al.,* 2009; Srisungnam *et al.,* 2009; Weng *et al.,* 2007). The TN sequence also served as template for the identification of single nucleotide polymorphism (SNPs) by comparison with genome sequence of a few other reference isolates. Three SNPs which yielded only four patterns (SNP1-4) and are further classified into 16 subtypes designated as SNP1A-D, SNP2F-H, SNP3I-M and SNP4N-P.The relative merits and information that can be generated from polymorphism at these two types genetic markers (STR &SNPs) have been databased (Monot *et al.,* 2008). The genetic diversity of *M. leprae* is very low which was successfully typed basing on short tandem repeats (STR) or microsatellites (repeat

length2-5 bp) and minisatellite (repeat length 6-50 bp) called as VNTR. Very fewer studies were described to validate strains of *M. leprae* by (TTC) 21 repeats. The genomic variation of *mycobacterium leprae* by (TTC) 21 repeats were first described by Shin *et al.,* Then Matsuoka *et al.,* described genotyping of *Mycobacterium leprae* on the Basis of the Polymorphism of (TTC) 21 repeats for the analysis of Leprosy Transmission.

Leprosy patients can carry out abundant number of bacilli and they casually spill out their bacilli to outside through skin, nasal ulcers and saliva (Davey *et al.,* 1974; Kotteswaran *et al.,* 1980). People who are close contact in their family have received droplet nuclei mainly by coughing and sneezing. Such leprosy patients can transfer the bacilli in a house or in a community who are in contact with households and neighbours and have other social relationships. The peoples who are close contact with leprosy have highest risk of contracting leprosy themselves. Risk with leprosy was estimated that leprosy was about nine times higher in household patients and about four times higher in neighbouring houses of patients compared with households that had no such contact with patients (Fine *et al.,* 1997; Van *et al.,* 1999). So this study in multicases families may be helpful to easy understanding of leprosy transmission. This method of study can notably explain persons in the family either affected with leprosy patient in the same household or with leprosy affected patient. The presents study involves in strain typing of multicase families thereby explore relationship in copy number of (TTC) 21 repeats among those families to easy understanding of leprosy bacilli transmission.

MATERIALS AND METHODS

Sample collection

LEPRA- India is a nongovernmental organization which was located 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, filariasis and eye care and it has research wing called The Blue Peter Public Health and Research Centre (BPHRC) which attain samples from the entire referral centres of India for the research purpose. So that in the present study we selected, 9 multicase families from BPHRC referral centre for VNTR analysis. The clinical finding, other relevant history was done by medical officer or physician and information was summarized in table-1 and table-2. From identified leprosy patients, the Slit Skin Smear samples were obtained with informed consent and normally sample collected areas were left ear lobe, right ear lobe and fore head with single 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 of multicase families were extracted through DNeasy Blood & Tissue Kit, Qiagen, Germany (cat No.69504) according the manufactures instructions, briefly ethanol soaked samples were further neutralized with phosphate buffer followed by ATL and proteinase –K treatment. The next step involves break down of cell wall and cell membrane, which was carried out by overnight incubation of sample at 55^0C then the sample was mixed with AL buffer and ethanol and heat at 70^0C for 20 min. Transfer the whole sample in to spin columns, and then washed the sample with AW1 and AW2 buffer .Finally the DNA was eluted in 50-100µL with AE buffer. The isolated DNA was stored in -4^0C until PCR was performed and for future use.

Amplification of (TTC) 21 repeats

The primers used for amplification of (TTC) 21 repeats were selected from earlier reports (Shin *et al.,* 2000). These selected primers were designated as (TTC) 21 -F 5′-GGACCTAAACCATCCCGTTT-3′ and (TTC) 21 -R 5′-CTACAGGGGGCACTTAGCTC -3′ ordered from Eurofins Genomics India Pvt. Ltd, Bangalore. Normally simple PCR were used to amplification of (TTC) 21 loci in designed population. The ingredients used and PCR cycling conditions were also obtained from previous reports(Shin *et al.,* 2000 and Sakamuri *et al.,* 2009) , briefly, 25µL of reaction mixture contains 12.5µL of red dye master mix, 8.5µL of nuclease free water, 1µL forward primer, 1µL reverse primer and 2µL of genomic DNA. The simple PCR conditions included , initial denaturation at 95°C for 10 min; 94°C for 90 s, 60°C for 90

s, and 72°C for 90 s min for 40 cycles ; finial extension at 72°C for 10min ; Termination and storage of PCR samples were kept at 4°C. The amplified products were further quality checked by 2% agarose.

Sequencing Analysis

The numbers of (TTC) 21 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 Vimta 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) 21 were read by counting of repeat units in the (TTC) 21 allele gene sequence.

RESULTS

SSC were obtained from a total of 9 leprosy cases after clinical manifestation and other relevant history was done and their patient history was summarized. Among all, 6 samples were MB cases with good bacterial load. The 78% cases were associated with nerve involvement and most of the cases were BT followed by BL cases. The TN strain was taken as reference to see the sensitivity, specificity and reproducibility of (TTC) 21. The primers used for the amplification of single (TTC) 21 region of 9 SSS samples, were successfully amplified and were detected in 2% agarose gel, the amplified product was identified as 200bp 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 all problems associated amplification

Table 1: General information and clinical classification of the patients including BI (BI-Bacteriological Index, M-Male, F-Female, TT-Tuberculoid leprosy, BT-Borderline Tuberculoid, BL-Borderline Lepromatous leprosy and LL- Lepromatous leprosy)

PHC code	ID No	Age	Sex	Clinical Classification	Nerves Involved	Ridley-jopling Classification	Average BI
AD	12/12	35	M	MB	Yes	LL	3.5+
AD	14/12	11	M	PB	No	BT	N
AD	17/12	11	F	MB	Yes	BT	1.5+
S3	003/12	50	F	MB	No	BL	2+
S3	004/12	48	M	MB	Yes	BL	2+
S3	005/12	15	F	PB	Yes	TT	N
S3	006/12	20	M	PB	Yes	BT	N
S3	021/12	48	M	MB	Yes	BL	4+
S3	022/12	24	F	MB	Yes	BT	2+

Table 2: General information and clinical classification of the patients including BI, (BI-Bacteriological Index-Male-Female, TT-Tuberculoid leprosy, BT-Borderline Tuberculoid, BL-Borderline Lepromatous leprosy and LL- Lepromatous leprosy)

PHC code	ID No	Age	Sex	Clinical Classification	Diagnosis date	Average BI
AD	01/12	42	F	MB	02/02/2012	2.25+
AD	02/12	23	M	PB	100/2/2012	N
AD	03/12	28	F	PB	22/02/2012	N
AD	004/12	40	F	MB	1/10/2012	1.75+
AD	05/12	57	M	MB	18/02/2013	3+
AD	06/12	60	M	MB	09/11/2012	2.75+
AD	07/12	41	M	MB	02/06/2012	2+
S3	08/12	17	M	PB	22/5/2012	N
S3	09/12	30	F	PB	28/5/2012	N
S3	10/12	48	F	PB	5/22/2012	N
S3	11/12	24	M	MB	22/5/2012	1+
S3	12/12	22	M	PB	22/5/2012	N
S3	13/12	29	F	MB	22/5/2012	4+
S3	14/12	51	M	MB	22/5/2012	2+

Enrollment of 9 multicase families having 14 cases with leprosy symptoms were screened by clinical manifestation. During the year 2012, 9 multicase families were identified and in which 14 house hold contacts were identified as leprosy by proper clinical identification. Among 14 cases 8 cases were male and 6 cases were female. The clinical finding revealed that 8 cases were identified as MB. The BI load was very high in MB cases were as in PB cases, the BI was negative. All cases were diagnosed in year 2012.The SSS samples were collected from all the identified leprosy patients and samples were stored in 70% ethanol until DNA isolation was performed.

Table 3: PCR and Sequencing results of representative samples (PCR=Polymerase Chain Reaction)

PHC code	ID No	PCR &sequencing repeat time	No.(TTC)21 repeat
AD	12/12	1	12
AD	14/12	3	13
AD	17/12	2	12
S3	003/12	1	10
S3	004/12	1	12
S3	005/12	3	16
S3	006/12	3	12
S3	021/12	1	10
S3	022/12	1	12

The multiple banding samples were further repeated to reproducibility of (TTC) 21 allele. We have seen the run to run analysis of the PCR and sequencing and identified as peak size and patterns remained stable. All PB samples having negative BI as well as MB samples with negative bacterial load were subjected repeated PCR and sequencing. Since less bacterial load, all the PB samples were showed hairy sequencing images; those were furtherer rectified by reproducibility. The PCR and sequencing results of 9 SSS samples were summarized in table-3. Due to the furry banding pattern on gel , the samples AD14/12 , S3N05/12 and S3N06/12 were repeated 3 times; AD17/12 were subjected to 2 times and rest of the samples due to high BI and good gel images were done for singe time. The samples which give good sequencing results within single repeat time were considered as MB. Most of the repeated units of (TTC) 21 were conserved and it followed previous reports from India. The repeated units of (TTC) 21 in AD12/12, AD17/12, S304/12, S306/12 and S322/12 were showed 12 times repeat of (TTC)21. The 10 times repeat unit of (TTC) 21 were observed in S303/12 and S321/12. AD14/12 and S305/12 have been identified as 13 and 16 units (TTC) 21 respectively.

Table 4: PCR and sequencing results of multicase families (PCR=Polymerase Chain Reaction).

PHC code	ID No	PCR & FLA repeat time	No.(TTC) 21 repeat
AD	01/12	1	12
AD	02/12	3	10
AD	03/12	3	12
AD	004/12	2	14
AD	05/12	1	10
AD	06/12	1	12
AD	07/12	1	15
S3	08/12	3	12
S3	09/12	3	10
S3	10/12	3	15
S3	11/12	2	12
S3	12/12	3	13
S3	13/12	1	13
S3	14/12	1	12

The total 9 families having 14 patients with leprosy symptoms and those were called as multicase families. Strain typing and molecular typing of leprosy in multicase families were very needful to control of the diseases in those families. The multiple banding samples were further repeated to reproducibility of (TTC)21 allele. All PB samples having negative BI as well as MB samples with negative bacterial load were subjected repeated PCR and sequencing. Since less bacterial load, all the PB samples were showed hairy sequencing images; those were furtherer rectified by reproducibility. The PCR and sequencing results of 9SSS samples were summarized in table-4. Due to the furry banding pattern on gel , the samples AD 02/12 , AD 03/12 , S3N 08/12, S3 09/12, S3 10/12 and S3 12/12 were repeated 3 times; AD 04/12 and S3 11/12 were subjected to 2 times and rest of the samples due to high BI and good gel images were done for singe time. The samples which give good sequencing results within single repeat time were considered as MB. Most of the repeated units of (TTC)21 were conserved and it followed previous reports from India. The repeated units of (TTC)21 in AD 01/12, AD03/12, AD 06/12, S3 08/12, S3 11/12 and S3 14/12 were showed 12 times repeat of (TTC)21 . The 10 times repeat unit of (TTC)21 were observed in AD 02/12, AD 05/12 and S3 09/12. AD 07/12 and S3 10/12 showed as 15 times of (TTC)21 repeats. Two samples that is S3 12/12 and S3 13/12 were identified as 13 repeats of repeats. Only one sample, AD 04/12 has identified as 14 times repeats of (TTC)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.,* 2010). 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 and Van *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 aim of the present study 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) 21 repeat allele gene which specify that *Mycobacterium leprae* is the only organism having (TTC) 21 repeat in *Mycobacterium* species. *Mycobacterium leprae* was tested for the stability (TTC) 21 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) 21 was good supportive to strain typing (Fontes *et al.,* 2009). 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 multicase family 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) 21 loci. Most of the VNTR and sequencing results were associated with high positive BI Multi Bacilli samples due to good concentration of DNA. The samples having low BI showed poor sequencing results which further repeated.

Earlier reports have been noted that >50% of leprosy patients in endemic country may have a history of intimate contact with an infected person (often a household contact) (Cherath *et al.,* 2006). In non

endemic places it can identify as 10% of leprosy patients may get disease from household contacts. Leprosy can transmitted by direct contact or by indirect way means through fomites. But it should due to long term contact of households with leprosy patients in their family. So our present study was therefore conducted to understand the transmission of infection within families with leprosy living in a rural endemic village. The present study was based on the detection of *M. leprae* from the slit-skin smears of index cases and their household contacts.

Present results from Indian patients were clearly indicated that (TTC) 21 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. Strain typing of leprosy from south Indian leprosy patients and North Indian leprosy patients were effectively carried out. Recently one more study from Mumbai, India was supported the above two studies and described as most of the repeat units are stable in all Indian patients (Mumbai). The present study was mainly based on strain typing of *Mycobacterium leprae* by Variable Number Tandem Repeats using specific prospective study design from multicase family leprosy affected individuals. Most of the samples showed 12 repeats of (TTC) 21 followed by 10 times. AD14/12 and S305/12 have been identified as 13 and 16 units (TTC) 21 respectively in representative samples. AD 07/12 and S3 10/12 showed as 15 times of (TTC) 21 repeats and two samples that is S3 12/12 and S3 13/12 were identified as 13 repeats of repeats in multicase families. Only one sample, AD 04/12 has identified as 14 times repeats of (TTC) 21 in multicase families. The present study explained the strain transmission of leprosy within families and other than families. AD 12/12, AD 01/12, AD 02/13 and AD 03/12 were single family and AD 12/12, AD 01/12 and AD 03/12 have possess same repeat number 12 which may suggest that AD 12/12 was carrier to AD 01/12 and AD 03/12 however AD 02/13 strain differ from the other strains from within the family. AD14/12, AD 04/12 and AD 05/12 were single family posses different copy number of (TTC) 21 locus which may suggest that AD 04/12 and AD 05/12 patient get infected with lepra bacillus from surrounding patients but not in the house hold patients. AD 17/12, AD 06/12 and AD 07/12 was from also single family in which AD 17/12 and AD 06/12 have same copy number 12 and AD 07/12 have copy number 15. S3 03/12 and S3 09/12 were from single family have associated with copy number 10.S3 04/12, S3 11/12 and S3 14/12 have coupled with single copy number 12 due single family. S3 05/12 and S3 13/12 have addressed with different copy number even though they were from single family. The 12 copy number was identified as single copy number in the S3 06/12 and S3 12/12. S3 21/12 and S3 10/12 composed of 10 and 15 copy number respectively which were from same family.S3 22/12, S3 08/12 were also associated with single copy number due to those are from single family. The patients within the single family exhibit same copy number which may suggested that, leprosy affected family number will transfer the diseases to all family members in the family. Different copy number of (TTC) 21 locus within the family may suggest that family members get infected with lepra bacilli from surrounding area not from same family member.

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