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ORIGINAL ARTICLE

Genotyping of *Mycobacterium leprae* strains in south central coast and central highlands of Vietnam

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ABSTRACT

Background and Objectives: Leprosy remains an important health problem worldwide. It is one of the oldest recorded diseases of humankind. In this study, we expanded the analysis of the geographic distribution of *Mycobacterium leprae* by investigating SNPs and *rpoT* genotypes in South Central Coast and Central Highlands clinical isolates, providing insights into the distribution and transmission of leprosy in Vietnam and in this geographic region.

Materials and Methods: 27 clinical isolates from the patients, determined the genotypes of *M. leprae* by SNP and *rpoT* polymorphism. SNP genotyping was performed by PCR amplification and sequencing, *rpoT* genotyping by PCR amplification and electrophoresis.

Results: All of 27 DNA samples (100%) were positive with RLEP TaqMan PCR (Ct value range is 18-32 on 3 replicates). SNP type 1 was identified in 15 isolates (56%), while SNP type 3 was detected in 12 samples (44%). SNP type 2 and type 4, were not detected. The 6-base repeat region of the *rpoT* gene was amplified by PCR and analyzed by 4% MetaPhor™ agarose gel electrophoresis. All isolates yielded amplification products of 91-bp, but not 97-bp.

Conclusion: This study showed that 56% of isolates belonged to type 1, 44% to type 3. In addition, all samples have the 3-copy hexamer genotype in the *rpoT* gene.

Keywords: Leprosy; *rpoT* protein; *Mycobacterium leprae*; Pseudogenes

INTRODUCTION

Leprosy is a chronic granulomatous infection of the skin and peripheral nerves with the intracellular bacterium *Mycobacterium leprae*, an acid-fast, rod-shaped bacillus. Leprosy remains an important health problem worldwide and is one of the oldest recorded diseases of humankind, reported for more than 2000 years (1). Leprosy is reported in various countries of the world and new cases of leprosy are still decreasing. In 2020, according to the WHO's report, 131 countries shared information on leprosy, accounting for 127,506 new cases and a registered prevalence of 129,303 cases. Most of the countries with high rates of detection of new cases are in African and South-East Asia Regions. Brazil, India, and Indonesia continue to report a major share of the leprosy burden in 2020 (2).

In Vietnam, the number of new leprosy cases in 2020 is 73 cases, and the new leprosy case detection rate per 1,000,000 population is 0.75. The number of leprosy patients under management is more than

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10,000 (3). Vietnam, along with four other countries (China, Malaysia, Papua New Guinea, and the Philippines), accounted for 86% of the total number of infections (4).

In China, there were only 521 newly detected leprosy cases in 2018. However, China still has the 22nd highest number of newly detected cases worldwide and the 2nd highest incidence in the Western Pacific region (5). A total of 464 new leprosy cases were reported nationwide in 2019 and a total of 2,219 leprosy cases were registered in China by the end of 2019 (6). In Korea, the number of registered patients has declined rapidly for 40 years (1977-2017) according to WHO statistics). Based on the WHO criteria, the number of registered (symptomatic) leprosy patients significantly decreased from 4,393 in 1997, to 166 in 2017. The number of new cases decreased from 39 in 1996 to 4 in 2017 (7). In India, new cases detected were 137,685 in the year 2007, and nine years later in 2016, the number remained almost the same at 135,485, a significant increase over the 127,326 new cases detected in 2015 (8).

Accurate and timely diagnosis of leprosy is critical for effective treatment and prevention of the spread of the disease. However, diagnosis of leprosy can be challenging due to the slow growth and low number of bacteria in clinical samples. Genetic techniques and genes have been developed to aid in the diagnosis of leprosy. PCR (polymerase chain reaction) is a genetic technique that can be used to detect the presence of *M. leprae* DNA in clinical samples, such as skin biopsies, slit skin smears, and nasal swabs. PCR can target various genes, including the RLEP gene, the 16S rRNA gene, the 18 kDa gene, and the gyrB gene (9). SNP (single nucleotide polymorphism) typing is a genetic technique that can be used to differentiate between different strains of *Mycobacterium leprae*. SNP typing targets specific SNPs in the bacterial genome that are unique to different strains. MLST (multilocus sequence typing) is a genetic technique that can be used to identify different strains of *M. leprae* based on variations in multiple genetic loci. MLST targets multiple genes, including the *folP1, gyrA, lepA, lepB,* and *rpoB* genes (10). VNTR (variable number tandem repeat) typing is a genetic technique that can be used to identify different strains of *M. leprae* based on variations in the number of repeated DNA sequences in certain genetic loci. VNTR typing can target various loci, including the RLEP locus, the SNP loci, and the STP loci (11).

Genotyping methods have been described for the molecular epidemiology of leprosy (12). Genetic variation in *M. leprae* has been investigated for the purpose of strain typing and ascertaining the geographical distribution of *M. leprae* strain types (13). Genotyping of causative agents of infectious diseases is essential for epidemiological analysis of transmission. 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 the historical and phylogenetic evolution of the bacillus that has affected humans and stigmatized leprosy patients for centuries (14). Some of the promising targets for eliciting strain differences among *M. leprae* are TTC (15), *rpoT* (16, 17), VNTRs (18, 19), and SNPs (14, 20).

The purpose of this study was to expand the analysis of the geographic distribution of *M. leprae* by investigating SNPs and *rpoT* genotypes in Central Vietnam clinical isolates. Comparing SNPs and *rpoT* genotypes with those from other Asian countries provided insights into the distribution and transmission of leprosy in Vietnam and in this geographic region.

MATERIALS AND METHODS

Samples. The slit-skin smears or punch biopsies were taken from leprosy patients primarily located on the south-central coast and central highland of Vietnam. A total of 27 samples were collected in this research. Samples were obtained from the skin lesions of patients. All samples were treated following the guideline of Kai et al. 2011 (21). The samples were soaked in 1 mL of 70% ethanol at room temperature in the field, before being sent to Quy Hoa National Leprosy & Dermatology Hospital laboratory. Biopsy specimens were kept at -20ºC until they were processed for DNA extraction.

DNA extraction. Total DNA was extracted from the samples using a DNeasy™ Blood and Tissue kit (Qiagen, CA, USA) following the manufacturer's instructions. The total DNA concentration and purity were evaluated using NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA). DNA samples were stored at -80ºC until use.

RLEP TaqMan PCR. The real-time PCR assay was performed by using forward primer (5'-GCAG-TATCGTGTTAGTGAA-3'), reverse primer (5'-CGCTAGAAGGTTGCCGTATG-3'), and the TaqMan probe (5' FAM-TCGATGATCCGGCCGTC-GGCG-TAMRA 3'), as described previously by Truman et al. which specifically target RLEP gene region used diagnosis of leprosy (22). The real-time PCR assay was profiled as follows: initial denaturation at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min in Mx3000P qPCR system (Agilent Technologies Inc, CA, USA). The DNA genome of *M. leprae* extracted from nude mice footpads provided by Leprosy Research Center (Tokyo, Japan) was used as a positive control. PCR was considered positive if the negative controls were all negative and a FAM signal with a cycle threshold value (Ct) of ≤37 was obtained.

SNP genotyping. To amplify three SNP loci, 1, 2 and 3 at nucleotide positions 14,676, 642,875 and 2,935,685, *M. leprae* genomic DNA (RLEP TaqMan PCR positive samples) was amplified using previously reported primer sequences (Table 1) (14, 20).

Five microliters of DNA extractions, 0.5 μM for each primer, 2.5 μL 2× Hot start PCR mix (LeGene Biosciences, USA) were combined in a 25 μL total volume reaction. PCR amplification was profiled as follows: initial denaturation at 95°C for 5 min; 40 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s; and final extension at 72°C for 10 min.

PCR products were purified and concentrated by using TM-5 kit (Zymoresearch, USA), following the manufacturer's instructions, and quantified on 2% agarose gel. To sequence both strands, two specific PCR primers were run for each sample. All samples were sent or sequenced at Sequencing Service (Faculty of Biology, Ludwig-Maximilians-Universität, Germany). Chromatograms were analyzed with Geneious Prime 2022.2.1 and compared with the reference sequence available in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) using the BLASTn plugin of Geneious Prime. All sequences were aligned using the ClustalX plugin of Geneious Prime (https://help.geneious.com/hc/en-us/articles/360044627352).

***rpoT* genotyping.** Primers A (5'-ATGCCGAAC-CGGACCTCGACGTTGA-3') and B (5'-TC-GTCTTCGAGGTCGTCGAGA-3') as described previously (16), were used for amplification to span the 91 or 97-bp fragment containing the target region with three copies of the six-base tandem repeats or four copies of the six-base tandem repeats in the *rpoT* gene. For comparing the differences of the repeats in the *rpoT* gene, 91- or 97-bp products were separated by electrophoresis in a 4% MetaPhor™ Agarose (FMC Bioproducts, Rockland, ME, USA) using 1× TAE buffer at 50 V.

RESULTS

RLEP TaqMan PCR. DNA was extracted from 27 samples, including slit-skin smears or punch biopsies, for real-time PCR diagnostics that targeted the RLEP region of *M. leprae*. All of 27 DNA samples (100%) were positive (Ct value range is 18-32 on 3 replicates). All negative controls were negative (Fig. 1). The RLEP TaqMan PCR permitted rapid analysis of batch samples with high reproducibility and is especially valuable for the detection of low numbers of bacilli. Molecular enumeration is a rapid, objective and highly reproducible means to estimate the numbers of *M. leprae* in tissues, and the application of the technique can facilitate work with this agent in many laboratories (22).

SNP genotyping. The PCR products of 3 fragments containing 3 SNP loci 1, 2, and 3 were checked by 2% agarose gel electrophoresis with low DNA mass™

Table 1. Primers for SNP genotyping of *M. leprae*

SNP loci	Primers	Amplicon (bp)
SNP14676	5'-AATGGAATGCTGGTGAGAGC-3'	194
	5'-CAATGCATGCTAGCCTTAATGA-3'	
SNP164275	5'-CTCGTCACAAATCCGAGTTTGAAT-3'	114
	5'-GTAGTAGTCTTCCAAGTTGTGGTG-3'	
SNP2935685	5'-ATCTGGTCCGGGTAGGAATC-3'	180
	5'-ACCGGTGAGCGCACTAAG-3'	

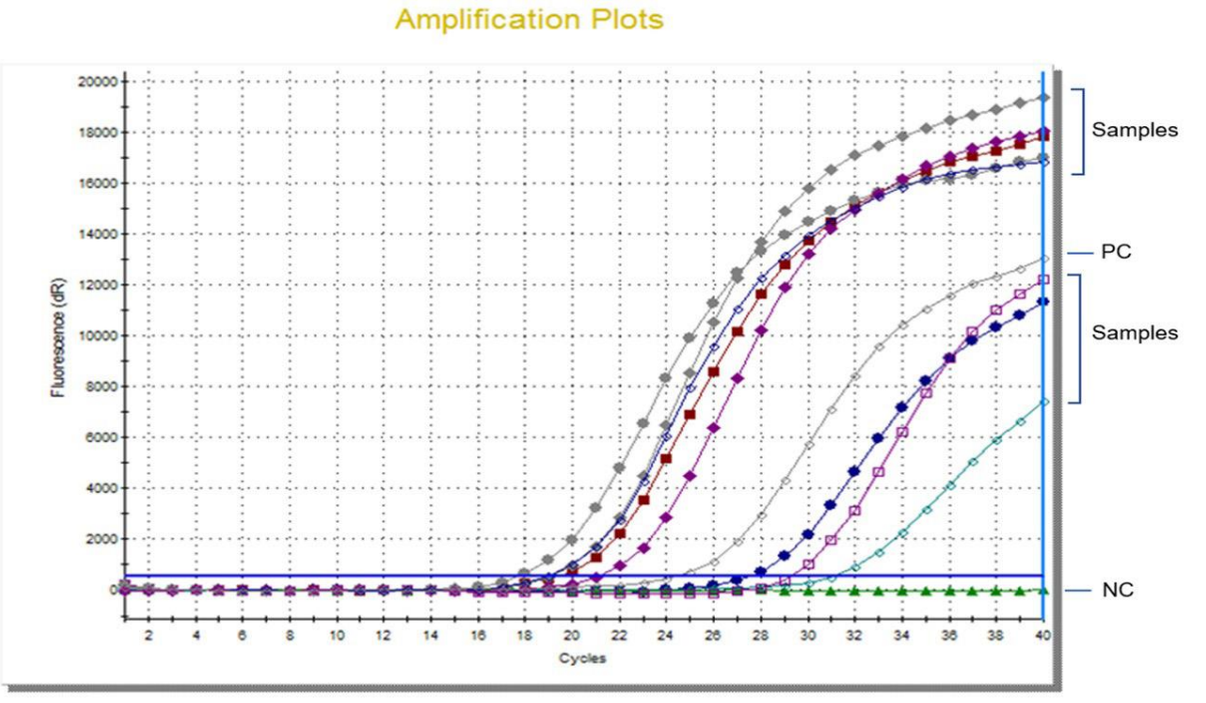

Fig. 1. Relative fluorescence and cycle number of RLEP TaqMan PCR. Samples: slit-skin smear/punch biopsies DNA; PC: Positive control; NC: non-DNA template.

Ladder (Invitrogen, USA) (Fig. 2).

The SNP type 1 was identified in 15 patients (56%), while the SNP type 3 was detected in 12 patients (44%). SNP type 2 and type 4, were not detected in our study. 18 of 27 samples (67%) locates the central Vietnam (include: Binh Dinh, Binh Thuan, Ninh Thuan, Khanh Hoa, Phu Yen, Quang Nam, Quang Ngai province), 9 of 27 samples (33%) locates in highland of Vietnam (include: Gia Lai, Kon Tum province) (Table 2).

Genotyping of the *rpoT* gene. The 6-base repeat region of the *rpoT* gene was amplified by PCR and analyzed by 4% MetaPhor™ Agarose gel electrophoresis. All isolates yielded amplification products of 91-bp, but not 97-bp (Fig. 3). SNP type 1 is predominant in Vietnam, Thailand, as it is in Myanmar. This pattern differs from that of other Asian countries, where SNP type 3 is more common than SNP type 1 (17, 23).

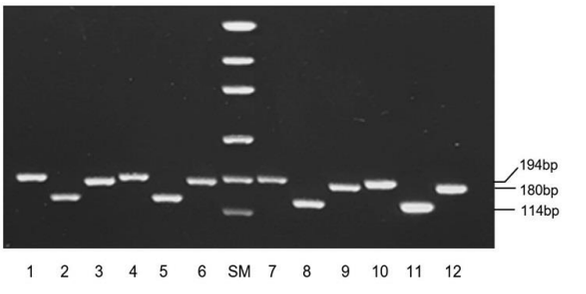

Fig. 2. Lane 1-12: amplicons of 3 fragments containing the SNP on 2% agarose gel electrophoresis. SM: 100bp DNA ladder (Invitrogen, USA).

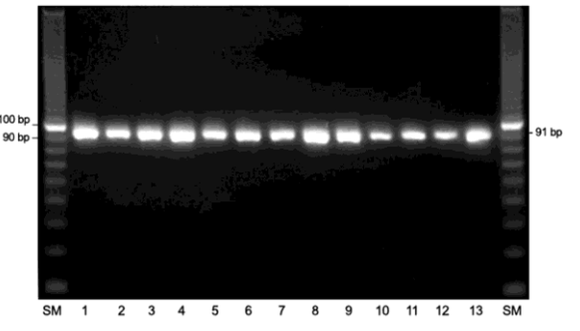

Fig. 3. Electrophoresis of 91 or 97 bp PCR products from three copies of 6 bp tandem repeats or four copies of 6 bp tandem repeats in the *rpoT* gene on 4% MetaPhor™ Agarose gel. SM: Low DNA Mass™ ladder (Invitrogen, USA)

DISCUSSION

SNP genotyping of *M. leprae* has also been demonstrated to have a characteristic worldwide geographic distribution and can be used to trace the worldwide dissemination of leprosy (14). The SNP types were

Table 2. The geographic distribution of SNP genotyping in the South-Central Coast and Central Highlands of Vietnam.

Sample ID	Areas	Location	SNP type
QH5		Binh Dinh	3
L109		Binh Dinh	1
L140		Binh Dinh	1
L551		Binh Dinh	1
L598		Binh Dinh	1
L665		Binh Dinh	1
L699		Binh Dinh	3
L802	South-Central	Binh Dinh	1
L831	Coast	Binh Dinh	3
L873		Binh Dinh	1
L829		Binh Thuan	1
L837		Binh Thuan	1
L410		Khanh Hoa	1
L454		Ninh Thuan	3
L595		Phu Yen	1
L654		Phu Yen	1
L491		Quang Nam	3
L839		Quang Ngai	1
QH3		Gia Lai	3
QH4		Gia Lai	3
QH64		Gia Lai	3
L158		Gia Lai	1
L660	Central	Gia Lai	3
L686	highland	Gia Lai	3
L807		Gia Lai	3
L153		Kon Tum	1
L285		Kon Tum	3

examined based on polymorphisms of nucleotides at positions 14676, 164275, and 2935685 of *M. leprae* genomic DNA. Four types of SNP- type 1, CGA; type 2, CTA; type 3, CTC; and type 4, TTC-were reported previously (14). And *rpoT* gene has a hexa nucleotide repeat and has been found to be present in three copies in most strains of *M. leprae* including the TN Indian strain but in four copies in eastern Asian countries such as mainland Japan and Korea (16, 17).

In 2007, the isolates from China belong to one (SNP type 3) of the four major types and represent an alternate eastward branch/route of transmission from the pro- posed origin in East Africa/Central Asia (24). But in the report in 2015, the majority of the leprosy strains were found to belong to SNP type 3, accounting for 78.8% (67/85) of the strains. Some leprosy strains belonged to SNP type 1, accounting for 20% of the strains (17/85). There was only one strain that belonged to SNP type 2, accounting for 1.2% (1/85) of all strains. None of the strains were found to be of SNP type 4 (25).

In the present group of isolates, all strains belonged to SNP type 1 and 3 at a ratio of 15:12. In addition, all samples have the 3 copies of 6-base tandem repeat in the *rpoT* gene. These results are almost similar to Thailand (23). Monot et al. 2005 previously described a classification of global isolates into four types based on the discovery of three SNPs. SNP type 1 *M. leprae* was shown to be prevalent in Asia (Nepal, India, South Korea, Thailand, and Philippines), the Pacific islands of New Caledonia, and East Africa, while type 3 was found in the European, North African, and American continents and also in New Caledonia (14). Matsuoka et al. compared *M. leprae* isolates from Japan and other Asian countries. So, can see that SNP type 1 and 3 frequencies and the *rpoT* 3 copy allele are common between Myanmar and the Philippines (17, 26), while the SNP type 2 was identified in Myanmar but not in Vietnam, Thailand or Philippine clinical isolates. *rpoT* 4-copy type can be found in Japan mainland, Korea, and Indonesia (16, 27).

These genotypes along with 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 (28). A combination of different typing systems is likely to be a more useful strategy (29). Our findings in Vietnam patients are consistent with previous studies demonstrating that repeats in *rpoT* and SNPs are considerably conserved, and this is useful for studying the geographic distribution of *M. leprae* in the region or at a global level.

CONCLUSION

This study detected the genotype and identified the common genotype of some *M. leprae* strains collected from Quy Hoa Dermatology Hospital, Binh Dinh province. Our analysis indicated that two genotypes, SNP type 1 and SNP type 3 exist in the South-Central Coast and Central Highland region of Vietnam, while SNP type 2 and SNP type 4 strains were not found. There were 15 strains that belonged to SNP

type 1, accounting for 56% of all strains. Some leprosy strains belonged to SNP type 3, accounting for 44% of the strains. In addition, all samples have the 3 copies of 6-base tandem repeat in the *rpoT* gene.

The study is limited by the relatively small sample size, as well as the fact that samples were only collected from the central and highland regions of Vietnam. In the future, genotyping *M. leprae* should be performed for all strains in Vietnam to improve our understanding of the molecular epidemiology of leprosy in Vietnam.

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