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Mycobacterium leprae Typing by Genomic Diversity and Global Distribution of Genotypes[1]

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Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* and is still a major health problem in the developing countries of Asia, Latin America and Africa ([28]). Global efforts to control leprosy by intensive chemotherapy have led to a significant decrease in the number of registered patients; however, new cases reported annually remain more than 0.5 million. The successful elimination of leprosy will likely depend upon a multifactorial approach, including appropriate chemotherapy, immunoprophylaxis and chemoprophylaxis. Critical to the success of any elimination strategy will be the identification and removal of the natural source of transmission and infection.

It has been difficult to identify sources of infection because of the protracted incubation period preceding clinical disease and because *M. leprae* remains uncultivable on artificial media. These two factors have slowed our understanding of the route of transmission of *M. leprae* and have continued to confound efforts to design strategies effective in eradicating the natural reservoir of *M. leprae* which could lead to the global elimination of this disease. Strain-specific markers could provide the necessary tools for understanding this aspect of the epidemiology of leprosy.

M. leprae appears to be minimally related to other mycobacteria based on the content of guanine plus cytosine, genome size ([3, 16]), unique biochemical features, such as phenolic glycolipid-I ([15]) and the presence of glycine in place of L-alanine in the peptide portion of peptidoglycan ([12]). Comparative studies of *M. leprae* isolates by total genomic hybridization have shown significant homology, suggesting genetic

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similarity among them ([2]). Related studies of the *M. leprae* genome using restriction fragment-length polymorphism (RFLP) have demonstrated that less than 0.3% of the nucleotides differed among the genomes in isolates of *M. leprae* from diverse origins, including leprosy patients, armadillos and mangabey monkeys ([4]). Williams, *et al.* reported that *M. leprae* isolates obtained from geographically distinct areas did not exhibit genotypic diversity by RFLP ([27]). de Wit and Klatser reported that isolates of *M. leprae* from different sources had identical 16S-23S rDNA intergenic spacer regions ([9]), a documented source of species and strain variability in some bacteria ([14, 18, 19]). In contrast, Fsihi and Cole demonstrated variability associated with the *polA* locus of *M. leprae* among eight chromosomal DNA samples originating from India, Africa and France ([13]). However, it was not clear whether diversity in the *polA* locus was applicable for typing of *M. leprae.*

Shepard and McRae have observed that the growth rate of *M. leprae* in mouse foot pads varies among isolates and that the differences are stable ([24]). Other studies have supported these results although the basis for phenotypes from the aspect of growth rate has not been elucidated. In an attempt to link growth rate with genotypic variation, we compared DNA sequences of various genes including *rpoT,* a homolog of the mycobacterial principal sigma factor ([5, 11, 23]). We found that a short repetitive region of *rpoT* exhibited a sequence polymorphism among isolates.

This report describes genetic diversity of the *rpoT* gene of *M. leprae* among 51 isolates obtained from 12 geographically distinct areas of the world and the distribution of genotypes.

MATERIALS AND METHODS

Source of *M. leprae* strains and preparation of genomic DNA. A total of 51 *M. leprae* isolates was used in this experiment. They were obtained from different areas of the world, and included clinical isolates from a biopsy specimen (BS) and those established by passages in nude mice (NM) or armadillos (AL). The countries from which *M. leprae* isolates were obtained and the number of isolates tested are as follows: Bangladesh (3 BS), Brazil (3 BS), Haiti (1 BS), India (1 AL), Indonesia (4 BS + 1 NM), Japanese Mainland (8 BS + 4 NM), Japan Okinawa (2 BS + 3 NM), Korea (11 BS), Nepal (1 BS), Pakistan (1 BS), The Philippines (1 BS + 1 AL) and Thailand (3 NM + 1 AL). Two of the *M. leprae* isolates were from the nonhuman origins of a naturally infected armadillo (Louisiana, U.S.A; 1 AL) and a mangabey monkey (Nigeria; 1 AL).

M. leprae was purified from tissues as described ([10]). Extraction of chromosomal DNA was carried out as previously described ([8]). Biopsy samples were homogenized, and partially purified bacilli were disrupted with freezing and thawing followed by phenol extraction and ethanol precipitation. For the preparation of bacterial DNA from biopsy specimens embedded in paraffin, three to five sections (5-μm thick) were treated by the method of de Wit, *et al.* ([8]). The skin-scraped materials were suspended in phosphate-buffered saline (PBS), purified partially by centrifugation, and DNA was extracted after freezing and thawing.

PCR and sequencing. PCR was carried out using Ampli Taq DNA polymerase (Perkin-Elmer Applied Biosystems, Norwalk, Connecticut, U.S.A.) in a 50 μl volume containing 150 pg of genomic DNA or 5 μl of template DNA solution and 1 μM of primers with reagents and protocols supplied by the manufacturer. Primers A (5′-AAGCGTCGATACAAAGGCACCGT-3′) and B (5′-AGTAGCTTCGCCATCCTCGGTTT-3′) were used for amplification to span the 300 bp fragment of the target region of the *rpoT* gene. The amplification was carried out in a thermal cycler (Astech PC800; Astech Co., Fukuoka, Japan) under the conditions of 30 sec at 95°C, 2 min at 60°C, and 4 min at 72°C for 30 cycles. The full length of the *rpoT* gene with up- and downstream regions was amplified with primers C (5′-GCTGTCGGTCACGGCTAT-3′) and D (5′-GAAAACCGCACCCCGATGGT-3′) under the conditions of 30 sec at 95°C, 2 min at 48°C, and 4 min at 72°C for 35 cycles. The short fragments, 91 bp or 97 bp, containing the target region of *rpoT* were obtained using primers E (5′-ATGCCGAACCGGACCTCGACGTTGA-3′) and F (5′-TCGTCTTCGAGGTCGTCGAGA-3′) under the same conditions as for the 300 bp fragment of the *rpoT* gene, except for 45 cycles of the thermal reaction. The same se-

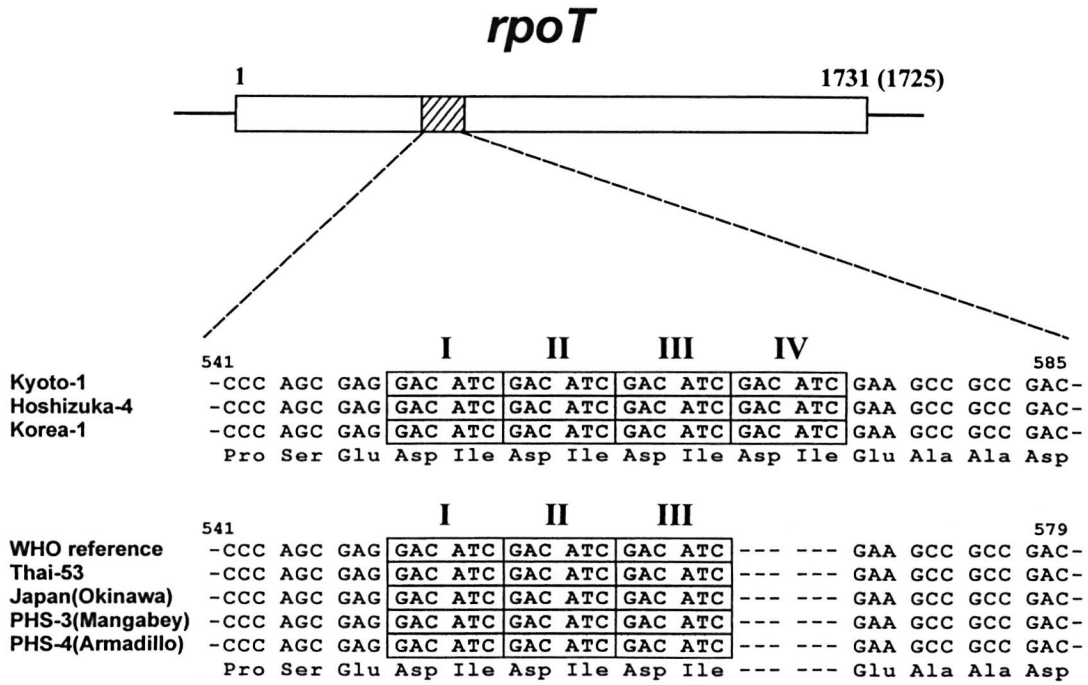

FIG. 1. Sequence alignment of DNA fragment corresponding from 541 to 585 (for 4 tandem repeats) or 541 to 579 (for 3 tandem repeats) of the *rpoT* gene from *M. leprae* isolates. Sequences from seven isolates and the reference (from Database, accession no. U15181) are shown. The nucleotide sequence data will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB019193 and AB019194.

quence information was obtained when EX Taq DNA polymerase (Takara Shuzo, Shiga, Japan), as 3′-5′ exonuclease activity, was used for amplification.

DNA samples for sequencing were recovered from agarose gel after electrophoresis using an Easy Trap DNA purification kit (Takara Shuzo). Determination of sequences was performed in both directions using a Thermo Sequenase kit (Amersham Life Science, Cleveland, Ohio, U.S.A.) with ^{35}S-dCTP or a BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Perkin-Elmer) in an ABI Prism. 310 Genetic Analyzer (Perkin-Elmer). The nucleotide sequences obtained were analyzed using the DNASIS computer program (Hitachi Software Engineering, Yokohama, Japan).

Agarose gel electrophoresis. For comparing the differences in the repetitive region of the *rpoT* gene, the short fragments of the target region were amplified, and reaction products, 97 and 91 base pairs, were electrophoresed in a 4% Meta Phor™ agarose gel (FMC Bioproducts, Rockland, Maine, U.S.A.) using TBE buffer at 75 volts.

RESULTS

Sequence variation in the *rpo*T gene of *M. leprae*. DNA sequences of various genes involved in replication, transcription and translation, such as the *rpoT*, the *rpoA*, the *tuf*, the *polA* and the 16S rRNA genes, were studied to determine whether DNA polymorphism exists in these genes that might explain growth rate differences in the mouse foot pad. A single sequence variation in the *rpoT* gene was observed among isolates but it did not appear to differentiate fast- and slow-growing phenotypes. Alignment of the 300 bp target region of the *rpo*T gene showed that the number of tandem repeats, composed of 6 bp repeats (GACATC), was different among the isolates. One group of isolates contained three tandem repeats; whereas a second group contained four repeats in the target region of *rpoT* (Fig. 1). Origins of *M. leprae* isolates from the latter group were limited to Japan (except Oki-

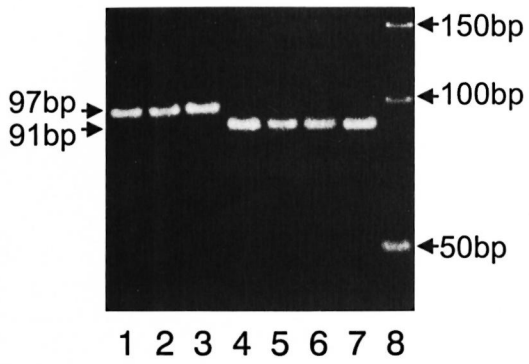

FIG. 2. Genotype detection by electrophoresis in agarose. Five μl of PCR products were electrophoresed in 4% Meta Phor™ agarose and then stained with ethidium bromide. Samples were: lane 1, Kagoshima (Japan); lane 2, Kanazawa (Japan); lane 3, Korea-1; lane 4, Thai-53; lane 5, Okinawa-1 (Japan); lane 6, PHS-3 (mangabey monkey); lane 7, PHS-4 (armadillo); and lane 8, the DNA size marker of 50 bp ladder.

nawa) and Korea, while isolates with three tandem repeats were found in the Okinawa Islands, southern Asian countries such as Bangladesh, India, Indonesia, Nepal, Pakistan, The Philippines and Thailand, and the Americas (Brazil and Haiti). In addition, two nonhuman isolates of *M. leprae* (armadillo and mangabey monkey) showed the three tandem repeat genotype.

Comparative analysis of the complete *rpoT* DNA sequence (1725 bp coding for 575 amino acids) among isolates of both genotypes showed that only the 6 bp tandem repeats were different. This analysis also included up- and downstream regions for a total of 1900 bp. To confirm that the strains used in this study were *M. leprae,* we identified the *M. leprae*-specific fragment of the *groEL* gene by the method of Plikaytis, *et al.* ([22]).

Genotype detection by electrophoresis in agarose gel. To simplify the detection of the two groups, we developed an assay capable of classifying the two genotypes. The assay was based on amplifying an approximately 90 bp region containing the *rpoT* target region and analyzing the mobility of resultant amplicons on 4% Meta Phor™ agarose. The DNA fragments obtained by PCR from two genotypes were clearly separated as shown in Figure 2.

DISCUSSION

The most striking finding was the apparent linkage of the four tandem repeat genotype between *M. leprae* strains isolated in Japan, excluding Okinawa, and those strains from Korea (Fig. 3A). The reason for this bias in distribution of the genotype with the four tandem repeat is not clear at present. A similar bias in genotype-specific distribution has been reported in *Helicobacter pylori* within Korean and Japanese populations. Epidemiological studies on *H. pylori* have indicated that almost all isolates from Korea and Japan are associated with the *cag*$^+$ genotype; whereas in other parts of the world 30% to 40% of *H. pylori* strains are *cag*$^-$ ([6, 17, 21]). In addition, *H. pylori* strains isolated from Asia could be separated from those of Caucasian origins on the basis of the nucleotide sequence ([1, 25]). It has been postulated that an *H. pylori* genotype-specific distribution is associated with human migration ([7]). Accordingly, it is reasonable to assume that the *M. leprae* strain-specific distribution that we observed may be related to patterns of human migration. Similarly, predominance of a single genotype was reported in *M. tuberculosis* in East Asian countries ([26]).

The *M. leprae* strains found in Okinawa were unrelated to the strains in the rest of the Japanese islands (Fig. 3A). There is a long history of concentrated interchanges between Korea and the main part of Japan, since the earlier migration of Mongolian people from Korea to Japan in the Yayoi and Kofun eras (300 BC to 600 AD). Several genetic markers have shown the close relationship between populations in Korea and most of Japan ([20]). In contrast, Okinawa was an independent country until the 19th century and had closer ties with southern Asian countries than with Japan.

The ubiquitous distribution of the three tandem repeat (Fig. 3B) suggests that further classification of this genotype will be necessary to distinguish strains of *M. leprae* for epidemiological studies. Fsihi and Cole reported genomic variability in a *polA* locus among five clinical isolates and three armadillo-grown strains of *M. leprae* ([13]). We examined this marker using eight strains of *M. leprae* (four strains with each *rpoT* type) according to their method but detected only one type of *polA*.

FIG. 3. Distribution of two genotypes of *M. leprae* isolates in Japan and Korea (**A**) and in the world (**B**). ● = *M. leprae* with three repeats in the *rpoT* gene; ○ = *M. leprae* with four repeats in the *rpoT* gene.

Despite efforts to detect genomic diversity among *M. leprae* isolates, clearly defined genetic polymorphism useful for epidemiological purposes has not been reported. Our results demonstrate the genetic diversity of *M. leprae* based upon differences in the *rpoT* gene of 51 isolates. Strains of *M. leprae* isolated were divided into two genotypes and their distribution in the world showed a striking bias, suggesting a relationship to the movement of the Korean and Japanese people. Further analysis of *M. leprae* strains is needed to explain the bias seen in the global distribution as well as to appreciate the utility associated with *rpoT* typing *M. leprae* for epidemiological survey.

SUMMARY

The genetic diversity and related global distribution of 51 *Mycobacterium leprae* isolates were studied. Isolates were obtained from leprosy patients from 12 geographically distinct regions of the world and two were obtained from nonhuman sources. Polymerase chain reaction (PCR) followed by DNA sequencing was performed targeting the *rpoT* gene of *M. leprae*. Isolates were classified into two groups based on the number of tandem repeats composed of 6 base pairs in the *rpoT* gene. Isolates from Japan (except Okinawa) and Korea belonged to one group, while those from Southeast Asian countries, Brazil, Haiti and Okinawa in Japan belonged to a second genotype. *M. leprae* obtained from two nonhuman sources (an armadillo and a mangabey monkey) revealed the latter genotype. These results demonstrate the genetic diversity of *M. leprae* and the related genotype-specific distribution in the world.

RESUMEN

Se estudió la diversidad genética y la distribución global de 51 aislados de *Mycobacterium leprae*. Los aislados se obtuvieron de pacientes con lepra de 12 regiones geográficas distintas del mundo y de dos fuentes no humanas. El análisis se hizo por la reacción en cadena de la polimerasa (PCR) y por secuenciación del DNA de la región correspondiente al gene rpo T de *M. leprae*. Los aislados se clasificaron en dos grupos con base en el número de "secuencias tandem" compuestas por 6 pares de bases en el gene rpo T. Los aislados de Japón (excepto Okinawa) y Korea correspondieron a un genotipo, mientras que los aislados de los países del sudeste asiático, Brazil, Haití, y Okinawa en Japón, correspondieron a un segundo genotipo. Los aislados de *M. leprae* obtenidos de dos fuentes no humanas (un armadillo y un mono mangabey) mostraron el sugundo genotipo. Estos resultados muestran la diversidad genética de *M. leprae* y la distribución de los genotips de *M. leprae* en el mundo.

RÉSUMÉ

La diversité génétique et la distribution géographique globale correspondante de 51 isolats de *Mycobacterium leprae* furent étudiées. Les isolats furent obtenus à partir de patients lépreux provenant de 12 régions géographiquement distinctes et deux isolats furent obtenus de source non-humaine. La réaction de polymerase en chain (PCR) suivie par le séquençage des produits obtenus fut réalisée sur le gène rpoT de *M. leprae*. Les isolats furent classés en deux groupes basés sur le nombre de motifs répétés 2 par 2 (tandem repeats) composés de 6 paires de bases au sein du gène rpoT. Les isolats du Japon (excepté Okinawa) et Corée appartenaient à un groupe, alors que ceux provenant de l'Asie du Sud-Est, du Brézil, d'Haïti et d'Okinawa au Japon appartenaient au second genotype. *M. leprae* provenant des deux sources non humaines (un tatou à neuf bandes et un signe mangabey) revélèrent ce dernier génotype. Ces résultats démontrent la diversité génétique de *M. leprae* et la distribution mondiale reliée au génotypes spécifiquement étudiés ici.

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