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Variable Numbers of TTC Repeats in *Mycobacterium leprae* DNA from Leprosy Patients and Use in Strain Differentiation

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Strain differentiation of *Mycobacterium leprae* would be of great value for epidemiological investigation to identify the infectious sources of leprosy, to understand transmission patterns, and to distinguish between relapse and reinfection. From the *M. leprae* genome sequence database, TTC DNA repeats were identified. Primer sets designed to amplify the region flanking TTC repeats revealed PCR products of different sizes, indicating that the number of repeats at each locus may be variable among *M. leprae* strains. The TTC repeats were not found in *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium marinum*, or human tissues, which indicated their specificity to *M. leprae*. Sequence analysis of the TTC repeat region in each of the *M. leprae* strains showed a variation of 10 to 37 repeats. In the *M. leprae* strains of 34 multibacillary patients at Cebu, Philippines, *M. leprae* with 24 and 25 TTC repeats was most common, and this was followed by strains with 14, 15, 20, 21, and 28 repeats. This study thus indicates that there are variable numbers of TTC repeats in a noncoding region of *M. leprae* strains and that the TTC region may be useful for strain differentiation for epidemiological investigations of leprosy.

Despite a rapid reduction in the number of registered cases over the last 15 years, leprosy is still a major public health problem in several countries (23). Even after the World Health Organization's (WHO's) efforts to eliminate leprosy as a public health problem by the year 2000, there remain hyperendemic areas in many countries which have shown no substantial decrease in the new case detection rate (23). In such areas, strain typing methods for *Mycobacterium leprae* would be of great value to identify the source of infection and to understand the transmission patterns of the organisms. In addition, strain differentiation methods for *M. leprae* isolates would be very useful in distinguishing relapse from reinfection after the completion of chemotherapy.

Several attempts have been made to identify polymorphic DNA sequences which could be used for *M. leprae* strain differentiation. However, restriction fragment length polymorphism (RFLP) analysis using various probes has not shown any differences between *M. leprae* isolates (4, 5, 16, 21). The single-strand conformation polymorphism patterns and DNA sequences of the region between 16S and 23S rRNA were also identical in *M. leprae* from different multibacillary (MB) leprosy patients (7). Although there are at least 28 dispersed repeats in the *M. leprae* genome (22), there are no reports on polymorphism based on the repeat. A recent report on a new class of *M. leprae*-specific repetitive sequence, RLEP, suggested another possibility for differentiating between *M. leprae* isolates, because PCR amplification of this repeat showed different intensities and the absence of the RLEP sequence in the pol(A) gene of certain *M. leprae* isolates (11). However, molecular typing based on this approach has not been fully explored in an effort to differentiate *M. leprae* isolates from different leprosy patients.

In the case of tuberculosis, RFLP analysis of *Mycobacterium tuberculosis* based on insertion sequences such as IS*6110* has been widely employed to understand organism transmission patterns and identify epidemics of highly virulent strains and multidrug-resistant *M. tuberculosis* strains (6, 18). However, RFLP analysis based on the Southern blot technique is not practical in *M. leprae*, mainly because of the difficulty of obtaining sufficient DNA to run RFLP since *M. leprae* cannot be cultured in vitro. PCR-based molecular typing of *M. leprae* targeting variable numbers of tandem repeats may thus be a more practical option in terms of applicability in clinical laboratories. In an effort to identify such variable numbers of DNA repeats in the *M. leprae* genome sequences available in the Mycobacterium DataBase, we focused on a TTC repeat in a noncoding region in the cosmid MLCB2407 (GenBank accession no. AL023596). In this study, we report evidence of variable numbers of this TTC repeat in *M. leprae* strains from MB leprosy patients.

MATERIALS AND METHODS

Biopsy samples. Biopsy samples were obtained from leprosy patients who visited the Skin Clinic at the Leonard Wood Memorial Center, Cebu, Philippines, before and 1 year after starting WHO multidrug therapy. As described previously (25), frozen 5-μm sections were prepared from each biopsy sample and stored at −20°C until used for PCR. Bacterial indices were determined both for biopsy samples and for slit-skin smears by microscopic examination.

For controls, peripheral blood mononuclear cells (PBMC) purified from five blood donors and a skin biopsy sample from a patient with secondary syphilis were included in this study. *M. tuberculosis*, *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium smegmatis*, *Mycobacterium phlei*, and *Mycobacterium marinum*, which had been maintained in the laboratory, were also included to examine the specificity of PCR.

Preparation of *M. leprae* DNA from biopsy specimens. *M. leprae* DNA was prepared from frozen biopsy sections as described previously (25). Briefly, about six frozen sections were disrupted vigorously in a microcentrifuge tube containing 100 μl of 0.1-mm zirconium beads in Tris-EDTA-NaCl (pH 8.0) and 50 μl of phenol-chloroform-isoamyl alcohol (25:24:1) using a bead beater (Biospecs Products, Bartlesville, Okla.) for 1 min. After centrifugation for 5 min, the aqueous phase was collected and mixed with an equal volume of chloroform-isoamyl alcohol (24:1). After another brief centrifugation, the upper phase was collected and boiled for 10 min to destroy DNase. DNA was then precipitated with ethanol and resuspended in 10 μl of distilled water before being used for PCR.

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TABLE 1. Number and frequency of TTC repeats in *M. leprae* strains from MB patients

No. of repeats	Frequency (no. of patients)[a]	GenBank accession no.
10	1	AF274483
13	1	AF274484
14	3	AF274485
15	3	AF274486
19	2	AF287975
20	3	AF274487
21	3	AF274488
22	1	AF287976
23	2	AF274489
24	4	AF287977
25	4	AF274490
27	2	AF274491
28	3	AF274492
32	1	AF274493
37	1	AF274494

[a] $n = 34$.

DNA from PBMC and other mycobacterial species was prepared by freezing in liquid nitrogen and boiling five times.

PCR amplification of TTC repeats. A 21-TTC repeat locus was identified in a noncoding sequence of the cosmid B2407 (GenBank accession no. AL023596), and a pair of primers was designed to amplify 201 bp flanking the entire 21 TTC repeats. The primer sequences were 5′-GGACCTAAACCATCCCGTTT-3′ (TTC-A) and 5′-CTACAGGGGGCACTTAGCTC-3′ (TTC-B). The PCR mixture (50 μl) consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 ml of $MgCl_2$, 0.2 mM deoxynucleoside triphosphate mix, 1 U of *Taq* DNA polymerase (Perkin-Elmer Biosystems, San Francisco, Calif.), 10 pmol of each primer, and DNA prepared from biopsy samples. After denaturing DNA at 94°C for 5 min, PCR was carried out for 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 10 min in a thermocycler (model 9600; Perkin-Elmer Co., Norwalk, Conn.). A 10-μl sample of each reaction mixture was run on a 3% agarose gel.

PCR amplification of the β-actin gene in PBMC was performed as described by Choi et al. (3).

Autoradiographic analysis of PCR products. The PCR mix (25 μl) consisted of 0.2 mM dATP, dGTP, and dCTP, 2 μM dTTP, 1.5 mM $MgCl_2$, 10 pmol of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.5 U of *Taq* DNA polymerase, and 0.5 μCi of [α-^{32}P]dTTP (3,000 μCi/mmol; NEN, Boston, Mass.). PCR was carried out for 30 cycles at the same conditions as above. Three microliters of PCR product was mixed with 3 μl of loading solution consisting of 95% formamide, 10 mM EDTA (pH 8.0), 0.05% bromophenol blue, and 0.05% xylene cyanol. The mixture was then denatured at 94°C for 3 min and loaded on 6% denaturing polyacrylamide gels with 7 M urea. Electrophoresis was carried out at room temperature for 4 to 6 h at a constant power of 50 mA. Autoradiography was performed for 12 to 24 h without an intensifying screen.

Cloning and sequencing of PCR products. PCR products from MB leprosy patients were purified using the GeneClean III kit (BIO 101, Vista, Calif.) and cloned into a PCR-TOPO vector in the TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.). The TOPO vectors containing PCR products were used for transformation of TOP10 competent cells (Invitrogen). Plasmids containing inserts were purified from broth cultures with the Qiagen plasmid kit (Qiagen, Inc., Valencia, Calif.) and sequenced with the AutoRead sequencing kit and ALF DNA sequencer (Pharmacia Biotech, Uppsala, Sweden).

Nucleotide sequence accession numbers. Nucleotide sequence data reported in this paper appear in the GenBank nucleotide sequence database under the accession numbers listed in Table 1.

RESULTS

PCR of TTC repeats. A pair of primers was designed to amplify the 201-bp region flanking the 21 TTC repeats in the cosmid B2407 (GenBank accession no. AL023596) of the *M. leprae* genome. In order to examine the specificity of the primer set, biopsy sections from leprosy patients and other mycobacterial species were used in PCR. When the PCR products were analyzed in the 3% agarose gel, only PCR products from MB leprosy patients gave a strong band in the 200-bp range (Fig. 1). On the other hand, biopsy samples from paucibacillary leprosy patients and DNA from other mycobacterial species did not show PCR amplification of DNA in this region. These results indicate that the primer set designed to amplify the DNA region flanking the 21 TTC repeats is specific to *M. leprae*. Interestingly, there were minor size differences in the PCR-amplified DNA from MB leprosy patients, suggesting a polymorphism in the PCR target DNA region.

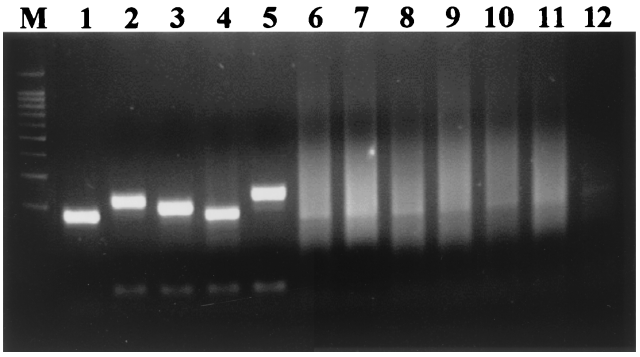

FIG. 1. PCR amplification of TTC repeats of *M. leprae* in biopsy samples from leprosy patients. Lanes 1 to 5, biopsies from MB patients; lanes 6 to 8, biopsies from PB patients; lane 9, *M. avium*; lane 10, *M. smegmatis*; lane 11, *M. phlei*; lane 12, healthy human PBMC; lane M, 100-bp size marker.

Since human chromosomal DNA contains TTC repeats, there is a possibility of DNA amplification with the primers used in this study. To rule out this possibility, PBMC from blood donors were used in the PCR amplification, with primers for the *M. leprae* TTC repeat region and primers for β-actin used as the control. As shown in Fig. 2, no PCR products were produced by these primers which corresponded to the *M. leprae* TTC repeat region, despite β-actin amplification in all cells of human origin. This result thus reconfirmed that primers designed to amplify the *M. leprae* TTC repeat region amplified specifically DNA of *M. leprae*.

M. marinum is known to cause a granulomatous skin disease. In order to rule out *M. marinum* in granulomatous lesions in the skin, DNA from the organism was used in PCR for amplification of the *M. leprae* TTC repeat region. As shown in Fig. 3, there was no amplification of the TTC repeat region. This indicated that there is no *M. leprae* TTC repeat region in *M. marinum*. In addition, there was no amplification of *M. leprae* TTC repeats in a biopsy sample from a syphilis patient. This result also reconfirmed that there would be little chance of

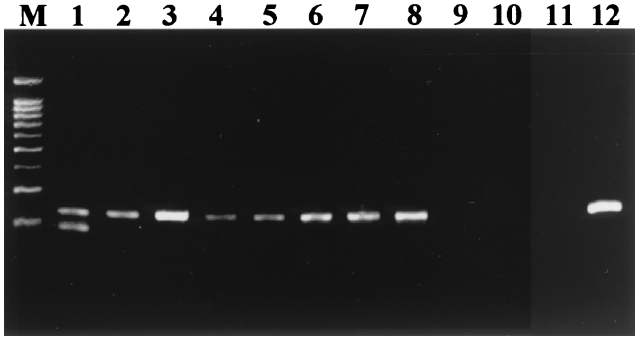

FIG. 2. PCR amplification of β-actin in biopsy samples from leprosy patients. Lane 1, multiplex PCR of TTC repeats and β-actin from the biopsy of an MB patient; lanes 2 to 5, biopsies from MB patients; lanes 6 to 8, biopsies from PB patients; lane 9, *M. avium*; lane 10, *M. smegmatis*; lane 11, *M. phlei*; lane 12, healthy human PBMC; lane M, 100-bp size marker.

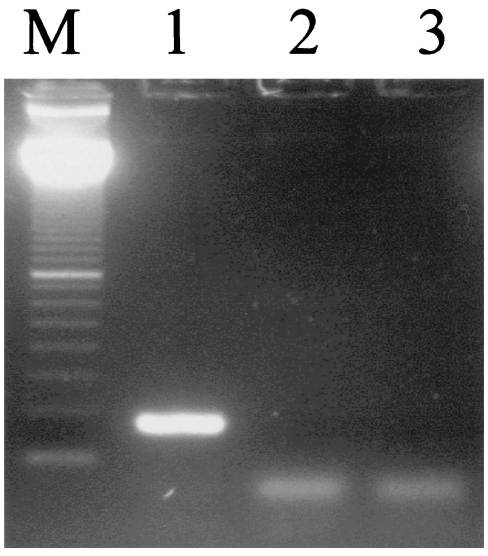

FIG. 3. PCR amplification of TTC repeat region of *M. leprae* in *M. marinum* and biopsy sample from a patient with syphilis. Lane 1, biopsy from an MB leprosy patient; lane 2, *M. marinum*; lane 3, biopsy from a syphilis patient; lane M, 100-bp size marker.

amplification of the *M. leprae* TTC repeat region in skin lesions from patients with nonleprosy skin diseases.

Autoradiographic analysis of PCR products. As suggested in Fig. 1, there might be minor differences in the sizes of the PCR-amplified products of *M. leprae* strains. In order to improve resolution and better visualize these differences, PCR was carried out using a PCR mix containing [α-^{32}P]dTTP, and PCR products were then denatured and run in a 6% polyacrylamide gel, followed by autoradiography. As shown in Fig. 4, there were marked differences in the size of PCR products between *M. leprae* strains from MB leprosy patients, which suggested that the target TTC repeat region may be a polymorphic locus of the *M. leprae* genome. In addition, a stepwise difference in size between the PCR products also suggested a difference in the number of TTC repeats between the *M. leprae* strains rather than random polymorphism in DNA sequences.

Sequencing of the TTC repeat region of *M. leprae* strains. In order to determine the DNA sequences of the TTC repeat region of each *M. leprae* strain, PCR products were cloned and sequenced using an automatic sequence analyzer. As expected from the autoradiographic analysis, the number of TTC repeats varied among the *M. leprae* strains, ranging from 10 to 37 repeats (Table 1). The nucleotide sequence data of *M. leprae* strains containing different numbers of TTC repeats were registered at GenBank, and accession numbers are listed in Table 1. Of 34 MB leprosy patients, there were 15 *M. leprae* strains with different numbers of TTC repeats; *M. leprae* strains with 24 and 25 TTC repeats were most frequent and were found in four patients, and these were followed by strains with 14, 15, 20, 21, and 28 TTC repeats.

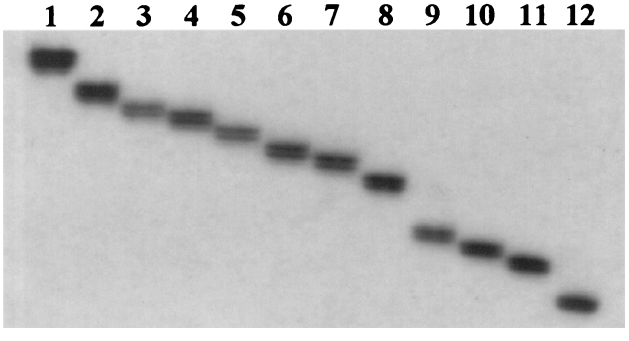

FIG. 4. Autoradiography of electrophoresis patterns of PCR products labeled with radioisotope of a flanking region of TTC repeats of *M. leprae* from leprosy patients. Lane 1, 37 copies of TTC repeats; lane 2, 32 copies; lane 3, 28 copies; lane 4, 27 copies; lane 5, 25 copies; lane 6, 23 copies; lane 7, 21 copies; lane 8, 20 copies; lane 9, 15 copies; lane 10, 14 copies; lane 11, 13 copies; lane 12, 10 copies.

Reproducibility of PCR and sequence analysis of TTC repeats. Since there were only small differences in the size of the PCR products of the TTC repeats, there may be technical problems in strain differentiation targeting the TTC repeat region. In order to examine the reproducibility of PCR and sequence analysis, biopsy specimens obtained from different sites at different times from four MB patients during chemotherapy were blindly analyzed by PCR, autoradiography, and sequencing after cloning the PCR products from the *M. leprae* strains. In all four pairs, the same number of TTC repeats was found in duplicate samples from each patient examined, indicating that PCR and sequencing of the PCR products were reproducible (data not shown). In addition, this implies that the TTC repeat seems stable during multiplication of *M. leprae* from infection to overt disease, which usually takes several years. These results thus indicate that the TTC repeats might be useful for the strain differentiation of *M. leprae*.

DISCUSSION

This study demonstrates clear evidence of sequence polymorphism in the TTC repeat region in *M. leprae* strains from leprosy patients. Such a variable number of short DNA repeats, known as a variable number of tandem repeats (VNTR), have been widely used for the molecular typing of both prokaryotic and eukaryotic cells (8, 10, 12, 13, 17, 19). For example, VNTR typing based on three, five, or six nucleotide repeats was useful in the epidemiological investigation of an outbreak by amoxacillin-resistant *Haemophilus influenzae* type b (17). Likewise, three nucleotide repeats, CAA/CAG, have been employed in the typing of *Candida albicans* strains (2). In addition, such short DNA repeats, also known as microsatellites, have been widely used in forensic investigation (15) and for determining the genetic traits of domestic and pet animals, including horses (1), pigs (26), chickens (14), and dogs (9).

Since TTC repeats are present in human chromosomes (24), PCR amplification of TTC repeats from normal human tissues had to be ruled out in this study. Firstly, both primers were designed based on the *M. leprae* genome sequences flanking the TTC repeats instead of TTC repeats themselves to make sure that only *M. leprae* genomic DNA would anneal with the primers. Secondly, specificity was verified by the lack of amplification of TTC repeats from the biopsy samples from paucibacillary leprosy patients, who usually have few *M. leprae* in their biopsy samples despite the presence of human tissues. Finally, DNA of PBMC did not give PCR amplification of TTC repeats despite amplification of the β-actin gene as a positive control for human chromosomal DNA. In addition, none of six mycobacterial species, including *M. tuberculosis* and *M. marinum*, gave PCR amplification of the TTC repeat region. Therefore, we are satisfied that the PCR products were specific to the TTC repeat region of the *M. leprae* genome.

Sequence analysis of the PCR products from each *M. leprae* strain of MB leprosy patients revealed differences only in the TTC repeats, and no differences between strains were shown in

the sequence between the TTC repeats and the primers in either direction. This suggested that most of the sequences in *M. leprae*, even in the noncoding region, are conserved, as has been shown in other studies (4, 5, 16, 21). The exact mechanism of the evolution of *M. leprae* strains with different numbers of TTC repeats remains to be explained, although a slippage or addition of one codon during replication is the most plausible explanation (20, 24). It is also not known yet how stable TTC repeats in *M. leprae* are during multiplication of its genome after infection in humans, which usually occurs several years before its clinical manifestations.

In the cosmid B2407, which was derived from *M. leprae* grown in armadillo, there were 21 TTC repeats. Interestingly, there were three leprosy patients who were infected with *M. leprae* strains with 21 TTC repeats (Table 1). Although the three patients were from Cebu City, Philippines, it is very hard to believe that there is any epidemiological association between *M. leprae* strains from patients in this study and those used in the preparation of the cosmid library. If there are other regions containing VNTR, a combination of VNTR including TTC repeats will allow easier strain differentiation of *M. leprae*. For example, there were at least five VNTR loci in *M. tuberculosis* (10), and they proved useful in determining evolutionary traits of *Mycobacterium bovis* and *M. bovis* BCG strains worldwide.

In previous studies, a polymorphic site in the *M. leprae* genome was searched for epidemiological investigation, particularly for distinguishing between relapse and reinfection. Using the molecular typing method, it is now almost possible to determine the difference between *M. leprae* strains. To our knowledge, this is the second piece of evidence which shows polymorphism in *M. leprae* strains, the first being the RLEP insertion sequences found in the poly(A) gene and described previously (11). However, PCR of the TTC repeats has an advantage over PCR of the RLEP insertion sequence because it can show the size differences of PCR products, whereas RLEP PCR shows intensity differences. It would be of great interest to compare the two polymorphisms using the same specimens.

In summary, the results of this study indicate that there are a variable number of TTC repeats in a noncoding region of the *M. leprae* strains and that the TTC region is useful for the strain differentiation of *M. leprae*, which could be used for epidemiological investigation and the PCR-based diagnosis of leprosy.

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