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Genotyping of *Mycobacterium leprae* on the basis of the polymorphism of TTC repeats for analysis of leprosy transmission

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The polymorphism of TTC repeats in *Mycobacterium leprae* was examined using bacilli from skin slit of leprosy patients attending at Central Special Skin Clinic, YGH and nasal swabs of their contacts to elucidate the possible mode of leprosy transmission. It was found that bacilli with different TTC genotypes were distributed among same household contacts and also harbored bacilli in patients were different TTC genotype from that harbored by the contacts. Genotypes of TTC repeats were found to differ between husband under treatment and his wife and also mother under treatment and her sons living in same house. These results revealed the possibility that in addition to exposure via the presence of a leprosy patient with a multibacillary (MB) infection who was living with family members, there might have been some infectious sources to which the residents had been commonly exposed outside the dwellings. A limited discriminative capacity of the TTC polymorphism in the epidemiological analysis implies the need of searching other useful polymorphic loci for detailed subdivision of clinical isolates. It was found that TTC genotype of bacilli harbored by household contacts was different with the TTC genotype by index cases. It was seen in the resident where TTC genotype was different in husband (index case) and his wife (HC) and the mother (index case) and her sons (HC). These results revealed that whether the family members get transmission either from their MB index cases or from outside the dwellings.

INTRODUCTION

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* infection. It has long being believed that the source of infection is untreated multibacillary leprosy patients. It has also been predicted that multidrug therapy (MDT) with strong bactericidal antibiotics (such as rifampicin) would reduce the source of infection and consequently interrupt further transmission to others. However, the number of new cases has shown no substantial decline. It is reported that about 600,000 to 700,000 new cases are continuously found in the world every year [1], which suggests that the transmission of leprosy bacilli still occurs, especially in countries of endemicity. Elucidation of the mode of transmission would be essential to reduce new cases detection rate. The differentiation of strains of leprosy bacilli by genomic polymorphism might be of great value in efforts to understand the mode of transmission of the disease. The range of molecular techniques for epidemiological analysis has expanded in recent years, and there are now many genotypic methods that allow a high level of discrimination between bacterial strains. Restriction fragment length polymorphism analysis, which is the method most widely

used for molecular epidemiology of tuberculosis, is not applicable for leprosy. *M. leprae* can not be grown in artificial medium, and almost no divergence was found by this finger printing assay [2]. Shin *et al*. discovered a genomic divergence of *M. leprae* by the variation of TTC repeats [3] and subdivided 34 isolates into 15 subtypes. Genotyping according to the TTC repeats for fragments amplified by PCR seemed to be feasible for molecular epidemiological analysis of leprosy transmission. A previous study by Saeki et al. revealed that *M. leprae* existed on the surface of nasal cavities of residents in areas of endemicity [4].

Here, we report the distribution of different TTC genotypes of *M. leprae* among family members of each household and inconsistent genotypes obtained from patients and their family members in the same dwelling. The results strongly supported the previously proposed hypotheses on the existence of an infectious source(s) other than that of patients living with family members.

MATERIALS AND METHODS

Samples from patients

To clarify whether the TTC genotype in one patient varies or not, genotypes of the bacilli obtained from various lesions of one patient were compared. Slit-skin smear samples from 45 lesions of 22 patients from Central Special Skin Clinic, YGH were obtained. Samples were collected in the same manner as is used for routine slit-skin smear testing for bacterial index examination.

The sample on the disposable surgical blade was soaked in 70% ethanol and kept at room temperature until use. The sample was removed from the blade and collected as a pellet by centrifugation at 10,000 rpm for 20 min in 70% ethanol and then washed with phosphate-buffered saline. The template was prepared by treatment with lysis buffer, and then the TTC genotype was examined.

Samples from patients and their contacts (who develop new case later) in the same dwelling

TTC genotypes of the bacilli from patients and their contacts (who develop new case later) living in the same dwelling were examined.

- Case 1 was MB case and his son developed as new case later.
- Case 2 was the same as Case 1 in another house.
- Case 3 was also MB case and after 10 months of MDT his daughter developed new case.
- Case 4 was also MB case and after 9 months of MDT his brother developed new case (Table 3).

Skin slit samples were collected from at least two lesions of each patient. The genotype of each isolate was examined as described below.

Samples from household contacts

TTC genotypes of the bacilli from nasal swab specimen of 100 household contacts (HC) were examined. HC were defined as persons sleeping during the night under the same roof. Nasal swabs were taken by introducing cotton tip swabs (sterilized *JCB* MENTIP, Japan) 2-3cm into each nostril successively, and rubbing gently on the lateral and median sides of each cavity. Swabs were immediately chilled and transported to the Immunology Research Division, DMR (L M) and analyzed.

Preparation of template DNA and sequencing analysis

Templates from nasal swab materials and slit-skin samples were prepared by treatment with lysis buffer at 60°C overnight as described in Klatser *et al*. [5], TTC repeat regions were amplified by PCR

with the primers indicated by Shin *et al.* [3]. Copy numbers of TTC repeats were examined by the direct sequencing of the PCR products. Briefly, the regions flanking TTC repeats were amplified using a G mixture and a FailSafe PCR system (EPICENTRE, Madison, Wis.). DNA samples for sequencing were recovered with a MinElute gel extraction kit (QIAGEN GmbH, Hilden, Germany) after electrophoresis of PCR products. Samples were sequenced with a BigDye terminator cycle sequencing FS Ready Reaction kit (Perkin-Elmer Applied Biosystems, Norwalk,Conn.) and an ABI Prism 310 genetic analyzer (Perkin-Elmer). The nucleotide sequences obtained were analyzed using DNASIS software (Hitachi Software Engineering, Yokohama, Japan). The forward primer was used in all sequencing reactions, since the nucleotide sequences of interest detected by the reverse primer were deduced to be identical with those detected by the forward primer.

Ethical approval

Informed consent was obtained from all subjects. The study was approved by the Institutional Ethical Review Committee of Department of Medical Research (Lower Myanmar). Bacillary samples of nasal swabs and slit-skin smears were collected after informed consent was obtained.

RESULTS

Genotype of the bacilli from the nasal swab samples

Of 92 dwellings, there were 18 houses in which 30 (33%) individuals carried the bacilli on the surface of their nasal cavities. Residents in these houses harbored different TTC genotypes from each other; their TTC genotypes were 9, 11, 12, 13, 14, 15, 16, 17, 21, and 22 repeats. The TTC repeats of the bacilli from the new MB case consisted of 11 copies, but the bacilli from his family contacts showed 14 and 17 copies. The TTC repeats of the bacilli from PB patient showed 21 copies but bacilli from his HC showed 15 copies. The TTC repeats of the bacilli from another new MB case consisted of 13 copies, but the bacilli from his family contacts showed 13, 16 and 9 copies (Table 1). Among the dwelling, the most predominant genotype was 16 copies of TTC repeats and the 2nd dominant type was 14 copies of TTC repeats (Table 2).

Table 1. TTC genotypes of *M. leprae* detected from the skin and surfaces of nasal mucosa of patients and surfaces of nasal mucosa from residents living in the same house

Sr. No.	Leprosy patients (Type)	Contacts (Relationship)	TTC genotype (Slit skin)	TTC genotype (Nasal swabs)
1	MB		11	15
2		Wife	-	14
3		Son	-	17
4	PB		21	18
5		Grandmother	-	15
6	MB		16	15
7		Son	-	16
8		Son	-	15
9	MB		13	16
10		Daughter	-	13
11		Daughter	-	16
12		Son		9

MB = Multibacillary
PB = Paucibacillary

Table 2. Frequency of each genotype observed in patients and household contacts

No. of repeats	Genotype frequency		
	Patient's lesions	Nasal mucus	Total
9	2	1	3
11	2	1	3
12	6	4	10
13	6	6	12
14	4	9	13
15	4	8	12
16	11	12	23
17	2	4	6
21	2	3	5
22	6	4	10
Total	45	52	97

Genotype of the bacilli in the lesions

From all 22 patients, 45 samples of different lesions showed identical genotypes. The most dominant genotype has 16 copies of TTC repeats in these patients. The other genotypes (number 9, 11, 12, 13, 14, 15, 16, 17, 21 and 22 copies of TTC repeats) were detected. The frequency of each TTC genotype observed in samples from lesions of the patients and the nasal cavities of the residents is shown in Table 2.

Comparison of TTC genotypes among patients in a dwelling

The TTC genotypes of *M. leprae* of index and secondary cases were compared. The genotypes of patients (index cases) and son (secondary case) harbored the bacilli with 13, 22, copies and 9, 17, copies of TTC repeats respectively in 2 household in this study. In case 3 who was MB case harbored bacilli with 11 copies of TTC repeats, after 10 months of MDT his daughter developed as secondary case and harbored bacilli with 14 TTC repeats. Another case 4 of household cases of two brothers showed different TTC genotypes (15 and 16 TTC repeats) within the family (Table 3).

Table 3. TTC genotypes of *M. leprae* obtained from household leprosy cases

Case No.	Patient (TTC genotype) in supposed index case	Patient (TTC genotype) In same HHC*
1	Father (13)	Son (9)
2	Father (22)	Son (17)
3	Mother (11)	Daughter (14)
4	Older brother (16)	Younger brother (15)

HHC* = House Hold Contact

DISCUSSION

Elucidation and understanding of the source and the routes of transmission of *M. leprae* are essential in developing measures to prevent an infection. Previous sero-epidemiological studies indicated wide-spread *M. leprae* infections within a population [6, 7, 8, 9], and studies by PCR on the distribution of the bacilli also found that many individuals in areas in which leprosy is endemic carried *M. leprae* on the surface of their nasal cavities [5, 4, 9].

These studies suggested the presence of an infectious source other than that of a patient within the same dwelling. The aim of this study was to clarify microbiologically whether or not MB cases in the same dwelling represent the main source of infection. Establishing a methodology to discriminate the isolates of *M. leprae* is fundamental for these purposes. Although many attempts have been made to subtype *M. leprae* isolates by genomic divergence [10, 11, 2], no useful methods for epi-demiological analysis have been developed. Recently two genomic polymer-phisms successfully discriminated isolates of *M. leprae* [12, 3]. One of the authors (M. Matsuoka) discovered that *M. leprae* isolates could be divided into two subtypes on the basis of the polymorphism in the *rpoT* gene.

The geographical distribution of each genotype in the world was biased and seemed to be related to prehistoric movement of the human race [12]. Nevertheless, the genomic diversity of the *rpoT* cannot be used for epidemiological tracing of the transmission of leprosy bacilli. Genotyping to compare diversity of short-tandem-repeat loci on the basis of PCR is feasible for molecular epidemio-logical analysis, since *M. leprae* is not cultivable and shows very low levels of diversion in genomic DNA [13]. Variety in the copy numbers of TTC repeats can be used to classify *M. leprae* into a considerable number of subtypes and discriminate isolates for each leprosy case.

It is reasonable to assume that if the index case in the same dwelling is the source of infection, the genotypes detected in the house should be identical among the

household members. In this study, various types of TTC genotypes were detected from nasal mucosa of the HHC.

However, our results clearly demonstrated that there were families with different TTC genotypes of *M. leprae* on the surface of nasal cavities among the residents in the same dwelling. Therefore, the results of the investigation suggest that these residents are contaminated by bacilli with different genotypes. No variations in genotype among the isolates obtained from various lesions in the same patient were shown. This result consequently enables comparisons of the genotypes of bacilli obtained from different patients.

We had identified the existence of TTC genotypes of *M. leprae* that differed between the newly detected family contacts and the supposed index case patient. These results strongly suggest that the bacilli did not originate from a single patient in the dwelling and also indicate the exposure of the family members to infectious sources out of the dwelling. Previous seroepidemiological studies suggested that for the majority of cases, the possible source of infection might be in the environment rather than in direct contact with leprosy patients [6,7,8]. The findings by PCR, which revealed the wide distribution of the bacilli among the residents in areas of endemicity, also indicated that the transmission of the bacilli was not only from the leprosy patients [5, 4, 9].

The present study strongly supports these assumptions respecting the infectious source(s). Although many epidemiological observations indicated that the household contact was the risk factor for the development of leprosy [14, 15], on the other hand, many new cases had unknown source of infection [14]. Therefore, the source of the secondary case is not only from his/her household. The tendency seen of the accumulation of patients in some families might be attributed to other conditions such as susceptibility to leprosy infection, which is related to genetic predisposition as well as to acquired factors [16]. Two groups of the household leprosy cases showed apparently different TTC genotypes between a father and his son, mother and daughter and among brothers.

The inconsistency of the genotypes between *M. leprae* isolates obtained from household cases of patients living in the same dwelling clearly indicates that these patients are not always the source in infections of the other family members. Though the members of the other groups of leprosy cases showed the same genotype, whether those people were truly infected by the patient in the house was unclear. The presence of the same genotype in two cases doesn't necessarily imply the infection was transmitted from a patient to family contacts, for some TTC genotypes, such as those of 10 and 13 repeats, were widely distributed in the areas.

Other polymorphisms which can discriminate within a given TTC genotype are needed to elucidate this problem. Better epidemiological analysis could be done by the combination of various genotyping techniques. However, TTC genotyping enabled the subtyping of *M. leprae* into more types than *rpoT* genotyping. It is expected that other short polymorphic-tandem-repeat loci exist in *M. leprae* genome, in similarity tothose observed in investigations of *M. tuberculosis* [17]. A combination with genotyping using other polymorphisms might be a useful tool for precise epidemiological analysis.

Other genotyping measures depending on other short-polymorphic-tandem-repeat loci are required. The frequency of 24 or 25 TTC repeats was the highest in the previous study, which examined *M. leprae* isolates obtained in Cebu, Philippines [3]. Bacilli with 10 copies of TTC repeats were most frequently isolated in the present study, and the bacilli with large numbers (such as 37) of TTC repeats were not detected (Table 3).

It is of interest to compare the frequencies of each genotype in different areas, since the

results of a previous study indicated that the spread of the bacilli with specific genotypes was consistent with migration of some human groups [12]. The evidence resulting from the present molecular epidemiological study indicated the existence of an infectious source other than patients in the same dwelling. Wide distribution of the bacilli among residents [5,4,9] and a high positive ratio of anti-PGL-1 antibody among healthy residents [7,8] suggested that the bacilli existed in certain sources to which people were commonly exposed. Taking these results into consideration, the environment seems to be the most likely infectious source. However, it has not been elucidated so far.

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