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Detection of *Mycobacterium leprae* by the polymerase chain reaction (PCR) in nasal swabs of leprosy patients and their contacts

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In the light of current leprosy control strategies, non-invasive samples such as nasal swabs may be more important than skin slit specimens as a source of material for epidemiological study. The objective of this research project was to investigate the use of a polymerase chain reaction (PCR) test to detect *M. leprae* in samples of nasal mucous from leprosy patients and asymptomatic household contacts of those patients. Nasal swabs from 15 paucibacillary (PB) and 55 multibacillary (MB) patients attending the Central Special Skin Center, Yangon General Hospital and 137 of their household contacts were tested for the presence of *M .leprae* by PCR and 33% of the samples of both patients and contacts were found to contain *M. leprae.* One of 32 (3.1%) swabs and 21 of 105 (20%) swabs were positive for *M. leprae* among contacts of PB and MB patients respectively ($p<0.05$). Among the patients, PCR positivity for nasal swab was 3 out of 15 (20%) in PB patients and 45 out of 55 (81.82%) in MB patients ($p<0.001$). Therefore, total 48 out of 70 (68.57%) in clinically diagnosed patients was PCR positive. PCR positivity of MB is significantly higher than PB in both patients and contacts. Although nasal carriage does not necessarily imply infection or excretion of bacilli, the finding of nasal carriage supports the theory of a disseminated occurrence of *M. leprae* in populations for which leprosy is endemic. This study is part of the molecular epidemiological study of leprosy in Myanmar.

INTRODUCTION

Following the introduction of the multidrug therapy (MDT) program in 1980, there has been a significant reduction in the estimated prevalence of leprosy worldwide, from around 12 million in early 1980, to approximately 1 million now. However, this decline in prevalence has not been mirrored by a concomitant fall in the observed incidence of the disease: over half a million new cases are still detected annually, a figure similar to that of 1985 [1]. This apparent discrepancy suggests that the widespread use of MDT is having little impact on the transmission of leprosy. Although the causative agent of leprosy, *Mycobacterium leprae*, has long been known, the exact mode of transmission of the disease remains to be fully elucidated [2]. There appears to be few natural animal hosts of this bacterium in endemic areas: human-to-human contact is, therefore, thought to play a major role in providing a reservoir of infection. In addition, the inability to culture *Mycobacterium leprae in vitro* makes assessment of subclinical infection rates difficult.

It is thought that the nose is the usual site of primary infection with *M. leprae*, as a result of airborne infection [3]. The advent of polymerase chain reaction (PCR) technology has afforded the opportunity to specifically detect small amounts of DNA, and a procedure, which indicate the presence of DNA equivalent to as few as 20 *M. leprae* cells, has been developed by Hartskeerl, *et al*. [4]. Studies using this technique have detected *M. leprae* DNA on

swabs taken from nasal mucosa of clinically normal individuals in family contacts of leprosy patients. The significance of the presence of such DNA is as yet unresolved, but may represent a form of sub-clinical infection or transient carriage of *M. leprae*, which may in turn be important in the transmission of the disease.

The technique described here is simple, sensitive, and specific for use in large-scale epidemiological studies. It can be used to monitor high-risk populations and also to maintain the achievements of leprosy elimination programs in countries where the disease prevalence has been significantly reduced. This study is part of the molecular epidemiological study of leprosy in Myanmar.

MATERIALS AND METHODS

Sample collection

Patients and contacts: After taken informed consent, nasal swab samples were collected from leprosy patients attending the Central Special Skin Center (CSSC), Yangon General Hospital and their family contacts. Fifteen PB and fifty-five MB patients with clinically and bacteriologically documented disease, as well as 137 household contacts (HHC) agreed to participate in the study. HHC were defined as persons sleeping during the night under the same roof. Leprosy patients were classified clinically and microscopically according to WHO classification [5] which consists of two categories, paucibacillary (PB) and multi-bacillary (MB). PB leprosy is defined as five or fewer skin lesions with no bacilli in skin smears, and MB leprosy cases have six or more lesions and may be skin smear positive.

Nasal swabs: 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 (Lower Myanmar) and analyzed.

Specimen preparation

The collected nasal swab samples were dipped in 1.5 ml eppendorf tubes containing 1ml PBS with 0.05% Tween 20 to release the bacilli from the cotton swab by turning and squeezing, then subjected to high speed centrifugation 14,000 rpm for 10 minutes. The supernatant was discarded and the sediment was resuspended in 1 ml 70% ethanol and prepared for DNA extraction by Klaster's Method [6].

DNA template preparation (DNA extraction)

DNA was prepared from nasal swabs according to the method of Klatser *et al* [6]. Briefly, nasal swabs immersed in 70% ethanol were centrifuged at 14,000 rpm for 10 minutes.After discarding the supernatant, the precipitate was washed with PBS and centrifuged again at 14,000 rpm for 10 minutes to remove remaining alcohol. The washed precipitate was suspended in 50µl of lysis buffer containing proteinase K 10mg/ml in 1M Tris-HCL, pH 8.5 and 0.5% Tween 20 and incubated at 60°C for 18 hours. Five microlitre of mineral oil was over-layed microlitre to prevent evaporation of water from the mixture. After heating at 97°C for 10 minutes, the suspended solution was treated with freezing and thawing twice to extract DNA and also to inactivate proteinase K, which inhibits Taq polymerase during PCR.

The DNA polymerase chain reaction (PCR)

A set of primers (5'-AAA AAA TCT TTT TTA GAG ATA CTC GAG-3' and 5'-CAA GAC ATG CGC CTT GAA-3') was used for amplification of the specific region of *M.leprae* 16S rRNA gene. *M. leprae* chromosomal DNA was kindly supplied by Dr. M. Matsuoka, Leprosy Research Center, NIID, Japan. This DNA served as a positive control in all PCR experiments.

The 50 µl reaction mixture contained 10 µl of template solution, 0.2 µl of *Ex Taq* DNA polymerase (Takara Shuzo Co., Shiga, Japan), 1 µM of each primer, 5 µl of 10x DNA PCR buffer, 8 µl of dNTP solution and 25.8 µl of water. The reaction mixture was overlayed with mineral oil 5 µl. The reaction was performed with a Mastercycler personal Eppendof AG, Hamburg, Germany. The reaction mixture was heated to 94°C for 1 min, rounds of amplification consisted of a 30 second denaturation step at 94°C, a 2 min annealing step at 44°C and a 3 min elongation step at 72°C for 45 cycles. The amplified DNA fragments were analyzed by electrophoresis on 1.5% (wt/vol) agarose gels in Tris-Borate EDTA (TBE) buffer.

RESULTS

A total of 70 leprosy patients (55 MB and 15 PB) attending the Central Special Skin Center (CSSC), YGH and 137 of their house hold contacts (HHC) were studied to detect *M. leprae* from nasal mucosa by PCR using the 16S ribosomal gene amplification. In 55 MB patients including both Bacillary Index (BI) positive and negative cases, 45 patients (81.82%) were PCR positive. Out of the 15 PB patients (BI negative) 3 cases were found to be PCR positive, that is 20% (Table 1). Table 2 shows 21 cases (20%) out of 105 contacts of MB cases were PCR positive and 1 (3.12%) out of 32 contacts of PB patients were PCR positive.

Table 1. Detection of *M. leprae* by PCR from nasal swabs in different types of leprosy

Types of Patients	PCR Results		Total
	Positive	Negative	
PB	3 (20%)	12 (80%)	15 (100%)
MB	45 (81.82%)	10 (18.18%)	55 (100%)
Total	48 (68.57%)	22 (31.43%)	70 (100%)

PB = Paucibacillary MB = Multibacillary
Pearson chi^2 (1) = 20.8988 Pr = 0.00001
Odds ratio = 18.0 95% CI =3.19-101.56

Table 2. Detection of *M. leprae* by PCR from nasal swabs in House Hold Contacts (HHC) of different types of leprosy

HHC in different types of patients	PCR Results		Total
	Positive	Negative	
HHC of PB	1 (3.12%)	31 (96.88%)	32 (100%)
HHC of MB	21 (20.00%)	84 (80.00%)	105 (100%)
Total	22 (16.06%)	115 (83.94%)	137 (100%)

HHC = House Hold Contacts, PB = Paucibacillary, MB = Multibacillary, Pearson chi^2 (1) =5.1812
Pr = 0.023, Odds ratio = 7.75, 95% CI =0.95-63

DISCUSSION

For more than a century, the excretion of *M. leprae* through the nasal mucosa of MB patients has been documented, and it represents the most important portal of exit for the organism. Whether *M. leprae* invades the human body after deposition of aerosolized organisms on the nasal mucosa is unknown. In the past, many attempts were made to detect *M. leprae* in Ziehl-Neelsen-stained smears from nasal swabs, but this technique is always open to criticism, particularly because of its lack of specificity.

PCR targeting species-specific sites of *M. leprae* DNA offers promise, in terms of both specificity and sensitivity. We investigated the household contacts of a number of PB and MB leprosy patients. Sampling of the nasal mucosa through swabbing is not the optimal technique because it is impossible to standardize. However, under field conditions it is the only practical possibility; a nasal washing procedure as performed by Shepard [7] would be impossible.

M. leprae was detected by PCR in 1 (3.12%) out of 32 samples from contacts of PB patients and in 21 cases (20%) out of

105 samples from contacts of MB patients. The difference is significant ($p<0.05$). Among the patients, PCR positivity for nasal swab was 3 out of 15 (20%) in PB patients and 45 out of 55 (81.82%) in MB patients. The difference is highly significant ($p<0.0001$, odds ratio=18.0, 95% CI=3.19-101.56). Therefore, PCR positivity of MB is significantly higher than PB in both patients and contacts. This might be an indication that carriage of *M. leprae* in the nose is indeed related to exposure to type of leprosy.

The results of this study provide evidence that a majority of MB patients are carrying *M. leprae* in their noses and that carriage of *M. leprae* occurs among healthy people living an area where leprosy is endemic. Nasal carriage by apparently healthy people might have an impact on leprosy control and thus be an important phenomenon from the public health point of view. New studies have to be undertaken to investigate whether and to what degree nasal carriage occurs in the general population of areas of endemicity and what might be the role of carriers in the maintenance of infection reservoirs and transmission of leprosy.

Our results differed from those published by Stefaan R, *et al* [8], who did not find a difference among contacts of PB and MB patients. The finding was 1 of 52 (1.9%) swabs and 13 of 164 (7.9%) swabs were positive for *M. leprae* among contacts of PB and MB patients. However, De Wit, *et al* [9] suggested that the percent positivity of the patients group was significantly higher than in both the group of occupational contacts and endemic controls. The amplification products were found in 55% of untreated patients, in 19% of occupational contacts, in 12% of endemic control, and in none of the non-endemic controls.

Although nasal carriage does not necessarily imply infection or excretion of bacilli, the finding of nasal carriage supports the theory of a disseminated occurrence of *M. leprae* in populations for which leprosy is endemic.

The technique described here is simple, sensitive, and specific for use in large-scale epidemiological studies. It can be used to monitor high-risk populations and also to maintain the achievements of leprosy elimination programs in countries where the disease's prevalence has been significantly reduced. This study is part of the molecular epidemiological study of leprosy in Myanmar.

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