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Detection of *Mycobacterium leprae* Nasal Carriers in Populations for Which Leprosy Is Endemic

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In order to better understand the role of *Mycobacterium leprae* nasal carriage in the maintenance of infection reservoirs and transmission of leprosy, we applied a polymerase chain reaction (PCR) that detected a 531-bp fragment of the *pra* gene of *M. leprae* on nasal swab specimens collected through a total population survey from individuals living in an area in which leprosy is endemic. Among the total tested population of 1,228 people, 7.8% were found to be PCR positive. PCR positivity was shown to be randomly distributed among the population for which leprosy is endemic. No association was observed between PCR positivity, age, or sex. The observed distribution of PCR positivity among households of different sizes confirmed the expected values, with the exception of two households, each with three people with PCR-positive nasal swab specimens. 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 problem of leprosy extends beyond the number of cases, estimated to be 5.5 million worldwide (14), since it involves severe handicaps, social stigma, and economic loss. Case finding and treatment are the generally accepted approaches to the control of leprosy. The implementation of the short-term multiple drug treatment, replacing the long-term dapsone monotherapy, has substantially reduced the prevalence of leprosy worldwide. However, the effect of treatment on the incidence of the disease, being the most important indicator of the progress of disease control, has not yet been validated (9).

Multibacillary patients are thought to be the main source of transmission of *Mycobacterium leprae*, the causative organism of the disease. However, if one plots the prevalence of lepromatous leprosy cases against total cases country by country, there is little correlation (13). The possibility that subclinical infections may be responsible for transmission has not been explored in detail. Considering the large number of bacilli carried by multibacillary patients upon diagnosis, it is likely that such patients may be infectious long before they are diagnosed. It has even been suggested that leprosy would arise from within a pool of subclinically infected people in a population for which leprosy is endemic rather than by transmission from an individual index case to new hosts (15). There is now indeed increasing evidence that the prevalence of infection exceeds that of clinical disease (18). If such an infected pool would play a role in the maintenance of infection reservoirs and transmission, its existence would have far-reaching consequences for the control of leprosy.

Leprosy is thought to be transmitted mainly aerogenically, and it is generally recognized that the nasal cavity is involved in the carriage and shedding of *M. leprae* (19). The nose is also considered to be one of the likely ports of entry of the bacilli.

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There is no easy and quick method for the reliable detection and identification of *M. leprae* in clinical samples. Detection of *M. leprae* through acid-fast staining and then microscopy lacks specificity and sensitivity. Although in vivo culture of *M. leprae* in mice is a possibility, this methodology is not suited for performance on a large scale. The polymerase chain reaction (PCR) is a novel, quick, and reliable method of detecting small numbers of organisms through the amplification of a species-specific DNA sequence to a detectable level (10). Previously, we developed a PCR for the detection of *M. leprae* based on the amplification of a 531-bp sequence of the *pra* gene (11). That PCR proved to be useful in the detection of small numbers of leprosy bacilli in skin biopsy specimens from patients with leprosy (6) and in nasal swab specimens from both patients with leprosy and their healthy contacts (5).

As a first step toward a better understanding of the role of *M. leprae* nasal carriage in the maintenance of infection reservoirs and transmission of leprosy, we applied our previous PCR for the specific detection of *M. leprae* DNA on nasal swab specimens collected through a total population survey from individuals living in an area in which leprosy is endemic.

MATERIALS AND METHODS

Field study. Two isolated adjacent villages in a rural area of South Sulawesi, Indonesia, with approximately 1,000 inhabitants each were selected for the survey. The leprosy prevalence was expected to be 5.9/1,000 and 0/1,000 population in the villages of Bantimala and Tondongkura, respectively, on the basis of the available information. At the time of the study, multiple drug treatment had not yet been introduced in the area, and the registered patients in Bantimala and Tondongkura all received dapsone monotherapy at irregular intervals. The villages were similar in terms of geographical, socioeconomic, and cultural conditions. The populations consisted mainly of traditional subsistence farm-

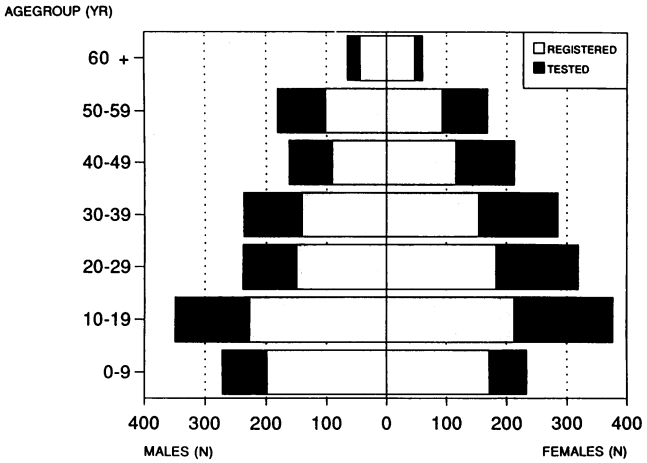

FIG. 1. Distribution of the registered and tested population by age and sex. Only specimens from people between 5 and 65 years of age were tested by PCR.

ers, with the majority of people living in wooden houses built on stilts. Facilities for drinking water and sanitation were poor. Before the study was undertaken, a total of 1,193 people were registered in Bantimala and 738 were registered in Tondongkura. Figure 1 shows the distribution of the population in the two areas by age and sex.

Before the study was undertaken, the populations of the villages were informed of the purpose of the study, and consent was obtained from all participants. Only residents living in the villages for at least 3 months were included in the study.

Clinical examination was carried out by experienced leprosy workers, and diagnosis based on the classification of Ridley and Jopling (16) was confirmed by the provincial leprosy medical officer (R.D.). Slit skin smears were taken from all patients for determination of the bacterial index. Household contacts of patients were defined as those persons living in the same house as the index case.

Pernasal swabs (Medical Wire and Equipment Co.) were used to collect nasal specimens. Specimens were not collected from individuals younger than 5 years of age and those older than 65 years of age. The specimens were collected by gently rubbing the swab several times over the inferior lateral conchae. The samples collected were coded, and PCR was performed without prior knowledge of the classification of the sample. All nasal swab specimens were stored at −20°C pending their analysis.

Preparation of specimens and PCR. The treatment of swab specimens with lysis buffer and PCR were done as reported before (5). The following is a summary. The specimens were subjected to PCR for the amplification of a species-specific 531-bp fragment of the *pra* gene of *M. leprae* and were subsequently analyzed for the presence of the amplification product by agarose gel electrophoresis. To confirm that each 531-bp fragment represented an amplification product from the *pra* gene region, hybridization with the 1.0-kb *Eco*RI fragment comprising the *pra* gene of *M. leprae* as a DNA probe was performed. To determine whether the sample contained inhibiting components that could have caused a negative result, samples in which a 531-bp fragment was not detected were submitted to a second PCR in which a 531-bp modified template was added to the reaction mixture (5). DNA was purified from the samples which were found to inhibit amplification of the modified template. These samples were run again in the PCR, with and without the modified template. The PCR run with the modified template served to monitor the effect of the purification.

PCR mixtures contained dUTP and uracil-DNA-glycosylase to prevent false-positive reactions because of cross-contamination with amplified DNA (5). In each run, positive controls of 375 pg, 75 pg, 15 pg, 3 pg, 600 fg, and 120 fg of chromosomal *M. leprae* DNA were included, as were five negative controls without target DNA (i.e., lysis buffer).

A specimen was considered positive when, with or without purification of DNA, a 531-bp fragment was revealed by both agarose gel electrophoresis and subsequent hybridization. A sample was considered negative when it did not show amplification in the first PCR and did not inhibit the amplification of the modified template. If a sample inhibited the amplification of the modified template, even after purification, we could not determine whether it contained *M. leprae* DNA or not. A total of 37 samples (2.9%) were excluded from the analysis because they were indeterminable.

Data analysis. All data were recorded on special forms and were managed with a data base and a statistical software package (version 5, Epi Info) in appropriate hardware. Analysis of variance methods were applied as indicated in the text. All probabilities presented are two-tailed.

To examine whether the distribution of PCR positivity was equal among all households, we compared the observed (O) and expected (E) number of PCR-positive individuals among households in a goodness-of-fit test, $\Sigma[(O - E)^2/E]$. The expected frequency of households with different numbers of PCR-positive individuals was calculated for each household by size by using the following formula: $(p \times q)^n$, where p is the overall PCR positivity rate, $q = p - 1$, and n is the number of people in the household tested. The expected number of households with each frequency of PCR positivity was then obtained by multiplication of the expected frequency with the number of households in each size category.

RESULTS

A total of 746 people in Bantimala and 556 people in Tondongkura were clinically examined; this represented, respectively, 69.3 and 84.0% of the registered population between 5 and 65 years of age. The male:female ratio in the examined population was 0.83, whereas it was 0.98 in the registered population (Fig. 1).

Of the 1,302 clinically examined individuals, 13 patients were diagnosed with leprosy, 8 in Bantimala (5 new and 3 old patients) and 5 new patients in Tondongkura, making the prevalence rates in these two villages almost the same: 10.7/1,000 and 9.0/1,000 population, respectively. Three of the 13 leprosy patients were females (23.1%), indicating a significantly lower prevalence of leprosy in females than in males (Table 1; Fischer exact test, $P < 0.01$). According to the clinical classification, four patients, two in each village, were diagnosed as borderline lepramatous (bacterial indices of the slit skin smears ranging from 1.0 to 3.5), 3 as borderline tuberculoid, and 6 as tuberculoid.

PCR was performed on the 1,265 samples that were collected, 729 in Bantimala and 536 in Tondongkura. Of those, 251 (19.8%) showed inhibition as established by PCR with the modified template. After DNA purification of these samples, 37 (2.9%) still showed inhibition and thus were indeterminable. They were excluded from analysis for this reason. A total of 165 negative controls divided over 33 PCR runs were examined. No false positivity was detected within

TABLE 1. Prevalence of PCR positivity and prevalence of leprosy by sex and geographic area

Sex	Area	No. of subjects	Prevalence of PCR positivity[a]		No. of subjects examined	Prevalence of leprosy[b]	
			%	No.		%	No.
Males	Bantimala	307	6.2	19	330	1.82	6
	Tondongkura	243	9.5	23	262	1.53	4
	Subtotal	550	7.6	42	592	1.69	10
Female	Bantimala	395	8.9	35	416	0.48	2
	Tondongkura	283	6.7	19	294	0.34	1
	Subtotal	678	8.0	54	710	0.42	3
Total		1,228	7.8	96	1,302	0.99	13

[a] PCR positivity rates did not differ between males and females or between the two villages (chi-square test, $P > 0.05$).
[b] The leprosy prevalence in females was significantly different from that in males (Fischer exact test, $P < 0.01$); no difference was found in leprosy prevalence between the villages (chi-square test with Yates correction, $P > 0.05$).

this group of controls. PCR results were not clustered by sample number, thus excluding bias in the PCR results because of sample collection (data not shown).

Among the total tested population, 7.8% (95% confidence interval, 6.3 to 9.3%) were found to be PCR positive. No age-related pattern could be revealed because the PCR positivity rates were similar for all age groups (chi-square test for trend, $P = 0.58$). Table 1 shows the prevalence of PCR positivity and the prevalence of leprosy in both villages by sex. There was no significant difference in PCR positivity between the two sexes or between the two villages (all combinations tested by the chi-square test; $P > 0.05$). On the basis of these findings, there was no need to use standardized PCR rates controlling for age and sex. Also, for the leprosy prevalence, only crude rates are shown in Table 1, since comparison of crude and standardized rates did not reveal any differences (data not shown). The leprosy prevalence in males was 1.7% and that in females it was 0.4%, while the PCR positivity rates in the two groups were 7.6 and 8.0%, respectively (Table 1). Clearly, the difference in occurrence of leprosy between the two sexes was not reflected in the PCR results.

Table 2 shows the PCR positivity rates for patients, contacts, and noncontacts. The positivity rate in patients was higher than those in contacts and noncontacts, but the difference was not statistically significant.

When PCR positivity rates among members within households of different sizes were compared, no difference was found (chi-square for trend, $P = 0.60$), indicating that the risk of being PCR positive was not related to the number of persons living together in one household. To examine whether the distribution of PCR positivity was equal among all households, we compared the observed and expected number of PCR-positive individuals among households (Table 3). PCR positivity was divided at random over the different households (chi-square, 21.76; degrees of freedom, 23; $P > 0.05$), with the exception of two households, each with three PCR-positive individuals. It is unlikely that this latter finding was due to chance (chi-square, 12.84; degrees of freedom, 1; $P < 0.001$).

DISCUSSION

The issues of the occurrence, distribution, and transmission of *M. leprae* infection are largely unresolved, but they are likely to be important for the control of leprosy. Through the application of PCR, it is now possible to detect directly and specifically the presence of *M. leprae* in large numbers of clinical samples. To detect nasal carriage of *M. leprae* in a population for which leprosy is endemic, we applied PCR on nasal swab specimens collected in a total population survey in two villages in an area in South Sulawesi, Indonesia, endemic for leprosy. The results of the study confirm that *M. leprae* nasal carriage is widespread among the general population in an area in which leprosy is endemic and is not restricted to a few patients.

One of the purposes of the present study was to compare PCR positivity rates between populations for which the endemicities for leprosy were different. In the village of Tondongkura, the prevalence rate was higher than expected, most likely because the multiple drug treatment leprosy control program was not yet implemented in the area. Furthermore, population-based surveys usually detect more patients than control programs only on the basis of passive case finding. Since the leprosy prevalences in the villages of Bantimala and Tondongkura were almost equal, no infer-

TABLE 2. PCR positivity rates for patients, contacts, and noncontacts

Status	No. of subjects tested	PCR positivity rate	
		No.	%
Patient	11	1	9.1[a]
Contact	42	1	2.4[a]
Noncontact	1,175	94	8.0
Total	1,228	96	7.8

[a] Not significantly different from noncontacts (Fisher exact test, $P > 0.05$).

TABLE 3. Distribution of the number of observed and expected PCR positive subjects among households

No. of PCR-positive subjects/household	No. of households		χ^2
	Observed	Expected	
0	277	274.3	1.16
1	70	74.6	5.46
2	10	7.4	2.28
3	2	0.2	12.84
Total	359	356.5	21.76

ences on the prevalence of PCR positivity in relation to the prevalence of leprosy could be made.

We found 9.1% of the patients to be positive by the PCR, which is less than the 55% PCR positivity rate in patients we reported before (5). However, in the previous study we only examined untreated multibacillary patients, while in the present study, patients with different classifications were included. Furthermore, a number of patients were on treatment with dapsone monotherapy while still having active leprosy. Treatment has previously been shown to greatly influence nasal carriage (3, 5).

PCR positivity was shown to be randomly distributed among the two populations. No association was observed between PCR positivity and age or sex. In addition, no difference in PCR positivity rates could be demonstrated between the two villages. This may reflect an equal risk for exposure to *M. leprae* independent of age and sex. Although the prevalence of leprosy was higher for males than for females, the PCR positivity rates did not reflect that difference. The excess of clinical leprosy among males, in view of the seemingly equal exposure to *M. leprae* on the basis of the PCR findings, might suggest a greater susceptibility of males to disease following infection rather than greater exposure to infection itself. On the other hand, we cannot exclude the possibility that the reported excess number of cases in males was partly due to ascertainment bias, since it is difficult to physically examine females completely in the face of the cultural barriers that exist in the area. However, the observed difference might be real because a male to female excess in leprosy has previously been reported in South Sulawesi (4).

We did not find a significant difference in PCR positivity rates between contacts and noncontacts. This is in accordance with a previously reported PCR study on nasal swab specimens (5). Evidence indicating that general populations in areas in which leprosy is endemic face similar risks of exposure to *M. leprae* also comes from seroepidemiological studies. The prevalence of antibody positivity to phenolic glycolipid I of *M. leprae*, which is thought to be a reflection of past or present infection, was shown not to be significantly different between contacts and noncontacts from several areas in which leprosy is endemic (1, 2, 17). Furthermore, isolated cases of leprosy in areas in which leprosy is nonendemic rarely lead to secondary cases of leprosy, despite evidence for transmission of infection (7).

Initial analysis of the data indicated that PCR-positive people were found in a selected group of households; i.e., 3.1% of the households tested were associated with 27% of all PCR-positive subjects. However, upon further analysis, controlling for household size, we found that the observed distribution of PCR positivity among households of different sizes confirmed the expected values. The only exceptions were two households each with three persons with PCR-positive nasal swab specimens. Although this finding was unlikely to be attributed to chance, the significance of it is as yet not clear. One can speculate that the members of these households were exposed to *M. leprae* to a greater extent than members of the other households in the villages were. Since these households were unrelated to the leprosy index cases, it is tentatively assumed that other sources of *M. leprae* infection exist in addition to some patients, such as subclinical multibacillary patients. A finding which favors the existence of other sources of transmission is the widespread distribution of *M. leprae* nasal carriage among the entire population for which leprosy is endemic. The existence of sources of transmission other than patients alone has been implied on the basis of epidemiological findings by others as well (8).

Asymptomatic carriage has already been suspected for a long time (8). Assuming that the specific and sensitive detection of *M. leprae* DNA through PCR indeed reflects the presence of bacilli, this is, to our knowledge, the first time that *M. leprae* nasal carriage has been specifically detected at the population level. Past experience with the PCR described here on a variety of samples from individuals with different forms of leprosy and from individuals from populations without disease for which leprosy is nonendemic suggests high specificity and sensitivity (5, 6). By including dUTP in the PCR in conjunction with uracil-DNA-glycosylase treatment of PCR products, false-positive reactions caused by contamination with amplified material were prevented. The effectiveness of this prevention system for this PCR has been shown before (5), and in the present study it was illustrated again by the negative results for 165 control samples which were run without template DNA. Use of a modified template in the PCR (5) enabled us to identify inhibiting specimens, which were then retested after purification of the DNA in the nasal swab specimens.

It is widely presumed that the prevalence of *M. leprae*-infected individuals exceeds that of individuals with clinical disease, mainly on the basis of immunological parameters (8, 18, 19). Here we reported a widespread *M. leprae* nasal carriage as detected by PCR among a population in Indonesia for which leprosy is endemic. Although nasal carriage does not necessarily imply infection or excretion of bacilli, the finding of it supports the hypothesis of a disseminated occurrence of *M. leprae* in populations for which leprosy is endemic. Widespread *M. leprae* infections in wild armadillos from areas in which armadillo leprosy is endemic has been reported before (12). Nasal carriage of *M. leprae* in healthy people may have important implications for leprosy control. It implies widespread exposure, which is difficult to envisage without the existence of sources of transmission other than multibacillary patients alone. Follow-up studies to determine the persistence of nasal carriage in individuals, currently in progress, may provide information on the significance of *M. leprae* carriage in the maintenance of infection reservoirs and the transmission of leprosy.

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