

## Nasal carriage of *Mycobacterium leprae* DNA in healthy individuals in Lega Robi village, Ethiopia

D. BEYENE<sup>1</sup>, A. ASEFFA<sup>1\*</sup>, M. HARBOE<sup>1,2</sup>, D. KIDANE<sup>1</sup>, M. MACDONALD<sup>3</sup>,  
P. R. KLATSER<sup>4</sup>, G. A. BJUNE<sup>5</sup> AND W. C. S. SMITH<sup>6</sup>

<sup>1</sup> Armauer Hansen Research Institute, Addis Ababa, Ethiopia

<sup>2</sup> Institute of Immunology, Rikshospitalet University Hospital, Oslo, Norway

<sup>3</sup> Department of Pathology, Institute of Ophthalmology, University of London, London EC1V 9EL, UK

<sup>4</sup> Department of Biomedical Research, Royal Tropical Institute, Amsterdam, The Netherlands

<sup>5</sup> Institute for General Practice and Community Medicine, University of Oslo, Oslo, Norway

<sup>6</sup> Department of Public Health, University of Aberdeen, Aberdeen, Scotland, UK

(Accepted 22 May 2003)

### SUMMARY

The number of registered leprosy patients world-wide has decreased dramatically after extensive application of WHO recommended Multiple Drug Therapy (MDT). The annual number of new cases has, however, been almost unchanged in several populations, indicating that the infection is still present at community level. Nasal carriage of *Mycobacterium leprae* DNA was studied in Lega Robi village in Ethiopia. MDT had been applied for more than ten years, and 718 residents over 5 years old were eligible for the study. During the first survey nasal swab samples were collected from 664 (92.5%) individuals. The results of a Peptide Nucleic Acid-ELISA test for *M. leprae* DNA interpreted by stringent statistical criteria were available for 589 (88.7%) subjects. Thirty-five (5.9%) individuals without clinical signs of leprosy were positive for *M. leprae* DNA. Seven PCR positive individuals lived in a household where one or two other members were also positive for *M. leprae* DNA. During a second survey 8 (4.6%) of 175 interpretable PNA-ELISA tests were positive. Of 137 individuals tested twice, only two were positive on both occasions whereas 10 were PCR positive only once. The study confirms the widespread distribution of *M. leprae* DNA in healthy individuals. The feasibility of curbing possible transmission of subclinical infection needs further consideration.

### INTRODUCTION

After extensive application of multiple drug therapy (MDT) for close to 20 years the number of registered leprosy patients has decreased dramatically. The number of countries showing prevalence rates above 1 per 10 000 of the population was reduced from 122 in 1985 to 32 at the beginning of 1998 [1]. In Ethiopia, a marked decrease in prevalence was seen from 1983

to 1996, with the curve then levelling off and a slight increase observed over the past 2 years [2]. Similar patterns have been observed in many other countries, e.g. from India and in the Western Pacific Region [3–5].

The incidence of infection with *Mycobacterium leprae* is difficult to assess since *M. leprae* has a long generation time and the infection develops slowly, often with an insidious onset of clinical symptoms. Indirect methods have to be used, and the number of new cases is often taken as the main indicator of disease burden [6]. The influence of diagnosis of ‘hidden

\* Author for correspondence: Armauer Hansen Research Institute, P.O. Box 1005, Addis Ababa, Ethiopia.

cases' needs careful consideration. 'Leprosy Elimination Campaigns' used quite extensively to promote MDT coverage [7] may reveal previously unknown cases, and the registered number of new cases may then be artificially higher than the true incidence of disease. In a Leprosy Eradication Drive study in Bombay it was indicated that a decreasing number of new cases may be detected following repeated drives [8], but the accumulated data indicate strongly that the incidence curves have remained flat in several populations extensively covered by MDT for more than a decade. The corresponding statement concerning leprosy trends 1985–97 in WHO's status report 1998 on the Action Programme for the Elimination of Leprosy [1] was 'at the global level, the leprosy prevalence rate was reduced by 85% and the new case detection rate by only 4%'. For a communicable disease such as leprosy, a decline in prevalence while the incidence curves are flat indicates that the infection is still transmitted at community level [5].

The mechanisms of transmission of *M. leprae* infection are unknown [9]. In a study of all known incident leprosy cases over 25 years in a high-endemic village in Indonesia, 79 (78%) of 101 cases could be associated to contact with another leprosy patient, and the highest risk of leprosy was associated with households of multibacillary patients [10]. Considering the large number of bacilli in multibacillary patients, it is likely that they are infectious long before they are diagnosed [11]. The relative importance of multibacillary cases for transmission may vary in different populations. Paucibacillary cases probably account for a significant part of the transmission in societies with a high frequency of this type of infection. Transmission of subclinical infection needs continued consideration [9, 11], and its significance may vary under different epidemiological conditions. The essential questions are how important is transmission rather than if it occurs, and in which circumstances chemotherapy based intervention strategies should be developed to curb transmission of *M. leprae* infection.

Since the main primary site of infection with *M. leprae* as a result of airborne infection is thought to be the nose [12], highly sensitive polymerase chain reaction (PCR) technology has been used to detect *M. leprae* DNA in nose swabs in various leprosy-endemic communities [11, 13–16].

The development and application of Peptide Nucleic Acid (PNA) technology [17, 18] combined with ELISA has been a central component in the Mucosal Immunity of Leprosy, MILEP-2 project [19]. The

purpose of the present work was to apply this PNA-ELISA technology to study nasal carriage of *M. leprae* DNA in healthy individuals in a leprosy-endemic village in Ethiopia in which MDT had been adopted for more than ten years.

## METHODS

### Study population

Lega Robi village was selected as the study area after consultation with the All Africa Leprosy Rehabilitation and Training Center (ALERT) Leprosy Control Supervisors who have extensive experience of leprosy work in Ethiopia. The village is located in Meta Robi Woreda in the highlands, 130 km north west of Addis Ababa, permitting sample transportation on a daily basis for storage and processing at AHRI. When the project was started in 1998, MDT had been in regular use in the area since 1984. The prevalence of leprosy in the district was 7.1/100 000 population. In the first quarter of 1998, 15 (1 paucibacillary and 14 multibacillary) residents of the district were receiving treatment for leprosy. Two of them were newly diagnosed in the quarter year.

Particular attention was given to establish a close link with the village leaders and the people based on the long time relationship of the ALERT Leprosy Control Supervisors with the village. The purpose of the study was explained and agreement for individual voluntary participation was established. The study received institutional and national ethical clearance. A detailed map of the village was then prepared prior to sampling with identification of the individual households and their inhabitants. The number of households was 182 and the number of residents 837, of whom 718 were over 5 years old and eligible for the study.

The first survey in Lega Robi was conducted from 11–14 August 1998. Samples were collected from 664 (92.5%) of the eligible individuals, and there were 47 (7.1%) registered villagers who did not show up for the examination and sample collection. None of the subjects from whom samples were collected had any signs of the disease.

The second survey in Lega Robi was conducted six months later. Nasal swabs were collected from 582 of 780 eligible residents. Of these, 284 were tested for *M. leprae* DNA and 175 passed the stringent quality control criteria (Table 1). The number of tests done after the second visit was lower because of logistic problems encountered during that part of the study.

Table 1. *Demonstration of M. leprae DNA by PCR in nasal swabs of apparently healthy individuals living in the leprosy endemic village Lega Robi, Ethiopia, 1998*

	Number of subjects					
	Total population	Eligible population	Sampled individuals	Tested individuals	Valid test results*	Positive
Visit 1	837	718	664	664	589	35
Visit 2	902	780	582	284	175	8

\* Test results meeting stringent quality control standards.

All subjects were asked whether they had been BCG vaccinated and were examined for the presence of a BCG scar on their deltoid area.

Flexible nasopharyngeal swabs (MW 160, Medical Wire & Equipment Co Bath Ltd, Corsham, UK), dipped in sterile saline immediately prior to use, were passed through the base of the inferior turbinate until the posterior wall of the nasopharynx was encountered. Swabs were collected, chilled and transported to the AHRI laboratory, and stored at  $-20^{\circ}\text{C}$  until analyzed for the presence of *M. leprae* DNA.

#### DNA extraction

The tip of the nasal swab was cut off and immersed in 100  $\mu\text{l}$  of lysis buffer: Proteinase K (Gibco/BRL-Life Technologies, Gaithersburg, MD, USA) 1 mg/ml in 100 mM Tris-HCl pH 8.5, 0.05% Tween 20. After incubation under mineral oil at  $60^{\circ}\text{C}$  over night in a water bath Proteinase K was inactivated at  $97^{\circ}\text{C}$  for 15 min.

#### Polymerase chain reaction (PCR)

The lysis buffer containing the tip of the swab was vortexed to ensure mixing prior to use for the PCR reaction. 50 ng of each primer was used for one PCR reaction with Ready-To-Go PCR Beads (Amersham Pharmacia, Freiburg, Germany) and 2  $\mu\text{l}$  from the lysed swab in a total volume of 25  $\mu\text{l}$  according to the manufacturer's instruction. The forward S13 primer 5'-CTC CAC CTG GAC CGG CGA T-3' and the S62 reverse primer generate a 531 bp amplification product [20] of 'the proline rich antigen', 'antigen 36', denoted *ML2395* in the *M. leprae* genome [21]. The reverse S62 primer 5'-Bio-GAC TAG CCT GCC AAG TCG-3' was biotinylated to bind the PCR product to the streptavidin-coated ELISA plate. The PCR programme was set as initial denaturation at  $95^{\circ}\text{C}$  for 5 min, followed by 37 cycles of  $94^{\circ}\text{C}$  for

2 min for denaturation,  $60^{\circ}\text{C}$  for 2 min for annealing, and  $72^{\circ}\text{C}$  for 2 min for extension with 10 min in the last cycle.

#### PNA-ELISA

The method was adapted from Egholm et al. [17] and Perry-O'Keefe et al. [18] as described previously [19]. Briefly, 10  $\mu\text{l}$  of each PCR reaction product was mixed with 18.4 ng of a fluorescein labeled PNA probe 5'-fluro-CCC AGC CAC GGT CCT-3' (Perspective Biosystems, Hertford, UK) in a final volume of 40  $\mu\text{l}$ . This probe hybridizes internally to the proline rich antigen PCR product. The mixture was incubated at  $95^{\circ}\text{C}$  for 10 min for denaturation and then cooled to room temperature for 2 h in a PCR machine for hybridization. The product was poured into a streptavidin-coated Immunol 2, 96-well plate (Boehringer-Mannheim GmbH, Mannheim, Germany), incubated for 1 h and washed 5 times with Tris buffered saline containing 0.1% Tween 20 (TBST). The plates were blocked with 2% BSA in TBST for 1 h at room temperature and washed 5 times. 50  $\mu\text{l}$  of horseradish peroxidase conjugated rabbit polyclonal anti-fluorescein antibody (Sigma, Gillingham, UK) diluted 1 in 2000 was added, incubated at room temperature for 1 h and washed as above. 50  $\mu\text{l}$  of the substrate orthophenyldiamine (OPD) at a concentration of 1 mg/ml in phosphate citrate buffer pH 5 containing 0.1% hydrogen peroxide (Sigma) was added and incubated for 15 min. The reaction was stopped by adding 50  $\mu\text{l}$  2 N HCl and the OD read at 492 nm in an ELISA reader (Titertek Multiscan Plus, EFLAB, Labsystems and Flow Laboratories, Helsinki, Finland).

#### Analysis of data

To ensure uniformity in presentation and validation of results, these were recorded based on criteria

Table 2. Household distribution of individuals with *M. leprae* DNA by PCR in nasal swabs at Lega Robi, Ethiopia, 1998

Number of positives per household	Household number observed	Household number expected	$\chi^2$	<i>P</i>	Degree of freedom
0	148	148.4	0.00		
1	28	27.5	0.01		
2	2	2.8	0.24		
3	1	0.2	2.73	0.0098	D.F. = 1
	179	179.0	2.99	0.39	D.F. = 3

described previously [19]. Briefly, for each analysis of test samples a common format was used, and the following acceptance criteria were applied to the results of the PNA-ELISA: (a) The mean OD for three negative controls should be  $<0.3$ , (b) the standard deviation in OD values of the negative controls should be  $<0.2$ , (c) the range (internal variation) between the OD values of the three negative controls should be  $<0.25$ , (d) the difference between the OD value of the highest positive control and the mean OD of the three negative controls should be  $>1.0$ . Four positive control reactions contained 1250, 125, 12.5 and 1.25 pg purified *M. leprae* DNA which was provided by Dr P. S. Brennan, Fort Collins, CO, USA, (e) the cut-off value for defining PCR positivity was defined by the formula: [Mean OD of negative control + (OD of highest positive control – mean OD of negative control)  $\times 0.3$ ].

### Statistical analyses

Data were recorded for each household on separate forms and entered into Epi-Info 6, version 6.04b statistical software package [22] for analysis. All significance test *P* values are two-tailed.

## RESULTS

A total of 182 households with 837 residents were registered during the first visit. The mean age of the population was  $20.9 \pm \text{s.d. } 17.1$  years and the median age was 17 years. Among these, 718 individuals aged 5 years or over were eligible for the study. Clinical examination was performed for signs and symptoms of leprosy. Nasal swab specimens were taken from 664 consenting individuals who had no evident or suspicious signs of leprosy (Table 1). All samples were processed by PCR and PNA-ELISA tests but the

results of tests on samples taken from 75 subjects failed the stringent quality control criteria and were therefore not included in the analyses.

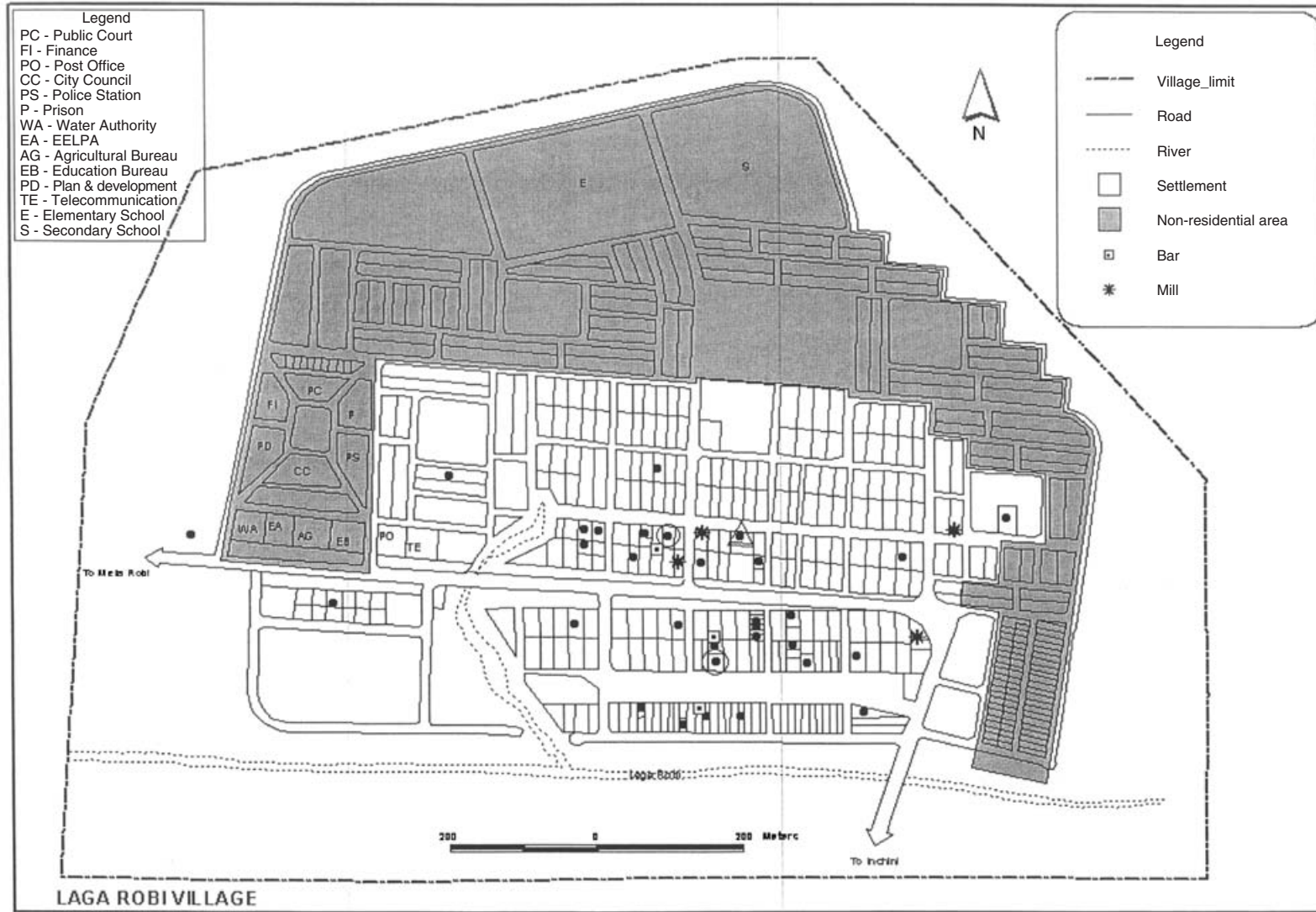
*M. leprae* DNA was confirmed in 35 (5.9%) of the 589 individuals screened in the first round. Of these, 16 were male and 19 female. The mean age of PCR positive subjects was  $26.4 \pm \text{s.d. } 19.5$  and  $25.3 \pm \text{s.d. } 12.7$  years for male and female, respectively. The corresponding figures for PCR negative subjects were  $24 \pm \text{s.d. } 17.3$  and  $23.9 \pm \text{s.d. } 15.2$  years. There was no statistically significant difference in nasal carriage of *M. leprae* DNA by gender or age.

The mean household size of Lega Robi village was  $4.6 \pm \text{s.d. } 2.7$  individuals. There were 31 (1.7%) of 182 households with at least one person positive for *M. leprae* DNA in the nose. Of these, one household had 3 positive members, and two households had two members. The remaining 28 had a single PCR positive household member. Multiple positivity within the same household was not related to sample collection or processing order. The mean household size was larger in households where *M. leprae* was detected compared to those households with no PCR positive members ( $6.5 \pm 3.0$  vs.  $4.1 \pm 2.4$ ). However, as shown in Table 2, the apparent association between household size and PCR positivity was not statistically significant. The geographical distribution of households with PCR positive members in the village is illustrated in Figure 1.

No association was found between a history of BCG vaccination and the presence or absence of *M. leprae* DNA in nasal swabs (Table 3). Similarly, there was no difference between subjects with or without a BCG scar with regard to nasal carriage of *M. leprae* DNA.

On a second visit to Lega Robi village six months later, a total of 902 subjects were registered. Nasal swabs were collected from 582 of 780 eligible subjects. Of these, 284 were tested for *M. leprae* DNA and





**Fig. 1.** Map of Lega Robi village showing distribution of households with members positive for *M. leprae* DNA in their nostrils. Dark dots indicate households with one member positive for *M. leprae* DNA by PCR. Two households (⊙) had two PCR positive members each and one household (△) had three PCR positive members. Shaded areas are non-residential parts of the village.

Table 3. *BCG vaccination status according to history or presence of scar and demonstration of M. leprae DNA by PCR in nasal swabs of healthy residents in Lega Robi, Ethiopia, 1998*

Vaccination according to	PCR results		Total	P value*
	Positive	Negative		
BCG history†				
Yes	0	29	29	0.40
No	34	519	553	
BCG scar‡				
Yes	0	17	17	0.61
No	34	521	555	

\* Fisher's exact test.

† Data inconclusive or missing for 7 individuals.

‡ Data inconclusive or missing for 17 individuals.

175 passed the quality control criteria. Eight subjects (4.6%) were found to be positive by PCR (Table 1).

When the results of the two visits are compared, 452 subjects were tested in the first round only. Among these 28 were found to harbour *M. leprae* DNA in the nose. Of 137 subjects screened during both visits, 5 were positive in the first round (but negative in the second), another 5 were positive in the second round (but negative in the first) and two were positive in both rounds. Nineteen subjects were tested only during the second round. One of these was PCR positive.

## DISCUSSION

To contribute to studies of leprosy epidemiology under field conditions in developing countries, techniques for collection of samples with subsequent transport and assay in more central laboratories have to be robust. This has been a central issue in the MILEP-2 project, and required the development of a combined PCR-PNA ELISA technology for demonstration of nasal carriage of *M. leprae* DNA as reported elsewhere [19] and based on an ELISA technique for rapid evaluation of a large number of samples for *Wuchereria bancrofti* infection [23]. The technique proved to be sensitive and applicable under these conditions. With sufficient sensitivity, it is essential to avoid false positive reactions. Samples from 219 healthy individuals in Norway, a leprosy non-endemic country, were therefore tested. All of them were negative, indicating a low risk of false positive reactions. After spiking of random samples with *M. leprae* followed by testing under blinded

conditions in London and India only spiked samples reacted positively in the PCR-PNA ELISA test further indicating its adequacy for the task [19].

Stringent criteria for data analysis were developed during a MILEP-2 project meeting in Amsterdam in January 2001 to ensure uniformity in presentation and validation of results obtained in different studies.

The percentage PCR positivity varied (3.3, 1.7 and 0.1) in three Indian villages studied using the same methods as in this report, namely Dongawardi, Shindawadi and Alkud respectively [unpublished data]. Our findings in Lega Robi are similar to those in Dongawardi. Although the percentage positivity varies, the presence of *M. leprae* DNA in healthy individuals in leprosy-endemic communities despite prolonged application of MDT shows a remarkable consistency, as was previously demonstrated in Indonesia [11, 15, 16] and the Philippines [20]. The significance of transiently positive patients, which was also observed in Indonesia [15], is unclear but seasonal variation in the occurrence of *M. leprae* was noted.

Among the 35 *M. leprae* DNA positive individuals in our first survey 7 lived together with one or two other positive members in the same household. Each of two households had two positives and one household had three PCR positive residents. Similar observations have been made previously. Klatser et al. [11] found two households in a village in Indonesia, each with three people with PCR-positive nasal swab specimens. Likewise, households with PCR positive members tended to be relatively more concentrated in certain parts of the village. However, this was not tested for statistical significance in this study. In a more detailed distribution analysis of PCR positivity rates in

Indonesia [15], the probability that positive individuals were clustered in certain small areas was over 90%. Adjacent areas were found to have a significantly higher positivity rate than expected.

One of the limitations of the present study is that surveys were not repeatedly done. The samples tested from the second survey were too few to yield satisfactory results. The number of positive subjects might have been inadequate to provide sufficient power for statistical significance when analysed for clustering. In addition, geographical coordinates were not obtained for statistical tests of spatial clustering in Lega Robi village. Nevertheless, the study provides evidence for the widespread distribution of *M. leprae* DNA among healthy people in Ethiopia and corroborates earlier reports by others that such PCR positivity in the individual is often transient.

The findings in the PCR based studies of nasal carriage of *M. leprae* DNA support the view that transmission of subclinical infection with *M. leprae* needs further consideration. The question remains of the significance of nasal carriage of *M. leprae* DNA and whether chemotherapy based intervention strategies should be developed to curb this transmission.

#### ACKNOWLEDGMENTS

We thank Village leader Ato Getachew Geda and his group for their assistance in organizing the village people to attend on time at our centre during sample collection. Nurse Desalegne Gobena, former Leprosy supervisor of the village is highly acknowledged for his help in enabling us to establish a link with the village leaders and the people based on his long time relationship with the village.

This study is part of the Mucosal Immunity of Leprosy (MILEP-2 study), supported by the European Union through Contract No. IC18-CT96-0047.

The MILEP-2 Study Group is as follows:

1. Smith CM, Smith WCS (Coordinator). Department of Public Health, University of Aberdeen, Aberdeen AB9 2ZD, Scotland.
2. Cree IA, Macdonald M. Department of Pathology, Institute of Ophthalmology, University of London, London EC1V 9EL, UK.
3. Klatser P, Oskam L, van Beers S. Department of Biomedical Research, Royal Tropical Institute, 1105 AZ Amsterdam, The Netherlands.
4. Harboe M, Beyene D, Aseffa A, Kidane D. Armauer Hansen Research Institute, P.O. Box 1005, Addis Ababa, Ethiopia.

5. Bjune GA. Institute for General Practice and Community Medicine, University of Oslo, Oslo, Norway.

6. Edward VK, Jadhav RS, Rao JR, Fernando A, Shinde V, Kamble R. Richardson Leprosy Hospital, Miraj, India 416410.

We thank Dr Ian A. Cree and Dr Rupendra S. Jadhav for advice during the work and Ato Girmay Medhin, Institute of Pathobiology, Addis Ababa University, for assistance regarding statistics.

The work was also supported by the Norwegian Agency for Development Cooperation (NORAD) and the Swedish International Development Cooperation Agency (Sida) through the core budget of the Armauer Hansen Research Institute.

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