The relation between seroprevalence of antibodies against phenolic glycolipid-I among school children and leprosy endemicity in Brazil

A relação entre soroprevalência de anticorpos contra o glicolipídeo fenólico-I entre crianças em idade escolar e endemicidade da hanseníase no Brasil

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ABSTRACT

Leprosy control programs would benefit expressively from an easy method to estimate disease prevalence and to assess the effect of leprosy control measures on disease prevalence. Determination of the seroprevalence of antibodies to PGL-I through school children surveys might be a useful indicator of leprosy prevalence at the district level. To investigate whether seropositivity rates could be related to leprosy detection rates and whether seropositivity could be used as a proximal indicator to predict the leprosy incidence in other areas, 7,073 school children in three different leprosy-endemic states in Brazil were tested. The results show a widely varying distribution of seropositivity in the communities independent of the number of leprosy cases detected. Seroprevalence was significantly lower at private schools. No differences in the patterns of seropositivity between ELISA and dipstick were observed. No correlation between leprosy detection rate and seropositivity rates could be established.

Key-words: Leprosy. Serology. Epidemiology. School children survey.

RESUMO

Os programas de controle da hanseníase se beneficiariam de um método fácil para estimar prevalência e avaliar o impacto das ações de controle na prevalência da doença. A determinação da soroprevalência de anticorpos contra PGL-I através de estudos com crianças em idade escolar foi sugerida como indicador útil da taxa de prevalência da hanseníase a nível municipal.Para investigar se a soropositividade estaria associada aos coeficientes de detecção da hanseníase e se poderia ser usada como indicador da prevalência em outras áreas, 7.073 crianças em três estados endêmicos de hanseníase no Brasil foram testadas. Resultados mostram uma considerável variação da distribuição de soropositividade nas comunidades, independente do número de casos de hanseníase detectados. A soroprevalência foi significativamente menor nos colégios. Nenhuma diferença na distribuição da soropositividade determinada por ELISA ou dipstick foi observada. Nenhuma correlação entre o coeficiente de detecção da hanseníase e soropositividade pôde ser estabelecida.

Palavras-chaves: Hanseníase. Sorologia. Epidemiologia. Estudo com escolares.

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Despite efforts to eliminate leprosy as a public health problem, the registered numbers of newly diagnosed patients worldwide increased from 571,792 detected cases in 1990 to 755,305 in 1998[20], representing an increase of 32% in the last decade. An optimistic explanation for the increase in the number of cases could be the contribution of active case investigation during leprosy elimination campaigns (LECs). Even so, attention must be directed to certain peculiarities of the pathogen and the disease to guarantee control and, possibly, the eventual elimination of the disease. Silent transmission of leprosy is facilitated by the slow growth characteristic of the bacillus, the long incubation period

before the disease becomes apparent, the slow progress of the irreversible nerve damage and the social stigma of leprosy.

Expertise regarding the diagnosis and treatment of leprosy will decline with the ongoing integration of vertical leprosy control programs into the general health care service[17]. Integration implies that less experienced professionals are expected to diagnose and classify leprosy. In communities where leprosy has been eliminated as a public health problem, less than one leprosy case would be diagnosed per every 10,000 people[18]. Most health professionals would never encounter a leprosy patient and might not include leprosy in the differential diagnosis if they believe it has been eliminated. Furthermore, the social stigma of leprosy would not necessarily be eliminated and stigma would continue to discourage patients from self-reporting. Additionally, when the socioeconomic situation remains unchanged in high endemic areas, susceptibility in the community will remain high. In leprosy-endemic areas where leprosy elimination is supposed to be achieved, the number of undetected leprosy cases may increase and silent transmission over the years could finally reverse the achieved status of leprosy elimination to one of even higher leprosy endemicity.

To evaluate whether the health care services are able to control leprosy, the control program must be monitored, but this presents certain challenges. The epidemiological indicator used to monitor the number of leprosy cases is detection rate and not leprosy incidence, because of the difficulty of detecting all cases. Active case finding surveys are not cost efficient for low incidence diseases, such as leprosy. There is currently no alternative simple way to establish leprosy endemicity in a particular area.

The detection of antibodies to *Mycobacterium leprae* is potentially useful for epidemiological studies of *Mycobacterium leprae* infection and could be useful for leprosy control programs[12 13]. Since the prevalence of seropositivity in a population roughly reflects exposure/infection rates, the effect of control measures could be evaluated by repeated serological screening[2 9]. Recently, it was shown that the determination of the seroprevalence of antibodies to PGL-I through school children surveys might be a useful indicator of leprosy prevalence at the district level in a leprosy-endemic area in Indonesia[16].

This study reports the results of an epidemiological study conducted in Brazil using the ML Dipstick to detect seropositivity among 7,073 school children in three different leprosy-endemic states. Our group investigated whether seropositivity rates are related to leprosy detection rates and whether seropositivity could be used as a proximal indicator to predict the leprosy incidence in a particular area.

MATERIAL AND METHODS

Study areas

Brazil is divided into 26 states and these states are divided into a total of 5,507 municipalities, which may vary extensively in terms of both area and population.

Three states in Brazil with different levels of leprosy incidence were selected for the study. The incidence of leprosy, estimated by the leprosy detection rate should be reliable and the areas should have well-established leprosy control programs. Therefore, the available epidemiological and operational leprosy indicators of the preceding five years were analyzed. The criteria for selection of the area and inclusion in the study were: (i) the presence of a well committed work team in the last 5 years, (ii) early diagnosis of new cases, and (iii) implementation of multi-drug therapy (MDT) in at least 90% of patients. To identify the study areas epidemiological data (detection rate, prevalence, and disability grade in new cases) and operational data (MDT program coverage, % of cases evaluated for disability grade and % of cured individuals among patients who completed treatment in the assigned period) were analyzed.

The states included were Espírito Santo (ES), Minas Gerais (MG) and Santa Catarina (SC), in which the general leprosy detection rates in 1998 were 4.1, 1.6 and 0.4 per 10,000, respectively. Based on the same criteria clarified above, in every state, three municipalities were selected, namely Aracruz (ES), Santa Teresa (ES), Colatina (ES), Governador Valadares (MG), Santa Luzia (MG), Barbacena (MG), Itajaí (SC), Tubarão (SC) and Laguna (SC). In each state, a project leader conducted the survey with the assistance of members of the local leprosy control programs from the Public Health System, schoolteachers and university students.

Study population

Children from the fifth class of primary school were studied, aiming at a target population of 10-14 year-old children. All procedures involving human subjects fulfilled the regulations of the local authorities and the Brazilian National Ethical Committee approved the project (619/99 REG CONEP: 806). Children for whom the parents gave written consent were clinically examined for signs of skin disease and whenever necessary, they were referred to the health center for treatment.

Sample size

The required sample size depends on the estimated prevalence of the characteristic to be studied and on the required precision of this estimate. In this study, the sample size calculations were based on an estimated seroprevalence and precision in low, medium and high endemic areas of 5% (± 3%), 15% (± 4%) and 25% (± 5%), respectively. The precision of the estimate was based on 95% confidence intervals. These expectations were based on a previous school children survey[16].

Since the method of cluster sampling was used, the required sample size also depended on the design effect, which is related to the number of clusters and the homogeneity of the characteristic within the clusters[3 15 19]. For practical and logistic reasons, a sample size of 750 individuals with a sampling design of 25 clusters of 30 individuals in eight municipalities and of 30 clusters of 25 individuals in one municipality was choose.

This design would confirm the estimated prevalence and required precision, while correcting for a design effect of 2 to 5 in the low endemic areas, 1.9 to 3 in the medium endemic areas and 1.3 to 4.2 in the high endemic areas.

The total number of children participating in all 9 municipalities from three states was 7,073.

Selection of clusters

For the selections of the *cluster schools*, a list of all schools in the municipality was prepared and schools were selected by sampling with probability proportional to size (PPS). This entails that the chance of being selected is not equal for every school, but is proportional to the size of the school. Depending on the size of the school, more *cluster classes* can be selected from the same school. Next, the fifth grade 5 to be included in the sample was selected at random. If there were not enough children in this class, another class was randomly chosen. In Santa Teresa and Laguna all the fifth grade classes were enrolled in the study due to the small number of children in the study population.

Blood collection

Approximately 10µl of capillary blood for direct use in the dipstick test was obtained from all participating children by finger-prick in a heparinized (capillary) hematocrit tube[5]. In addition, 3 drops of blood were collected on cards of 3 by 5cm of Schleicher & Schuell blotting paper GB 002. On each of these cards, a personal identification label was placed and the name, date of birth and date of blood collection were recorded. The cards were air-dried and then stored in zip-seal plastic bags at + 4°C.

Dipstick assay

The dipstick assay for the detection of antibodies to PGL-I of *Mycobacterium leprae* was prepared as previously described[6]. The dipsticks have two bands: an antigen band consisting of the *Mycobacterium leprae*-specific and immunodominant disaccharide epitope of PGL-I linked to bovine serum albumin (Natural Disaccharide-BSA or ND-O-BSA)[8] and an internal control band, consisting of anti-human IgM antibodies that bind to IgM molecules from the serum. The IgM detection reagent consists of a lyophilized monoclonal anti-human IgM antibody linked with a colloidal dye. Briefly, dipsticks were wetted in distilled water for 15 sec and then incubated for 1h in a reaction vial containing the 200µl of the reconstituted detection reagent and approximately 10µl of heparinized whole blood[5]. At the end of the incubation period, the dipsticks were rinsed with tap water and air-dried at ambient temperature. A reddish stained antigen band indicates a positive reaction. The results were scored as positive when staining was observed; no coloring (but with a positive control band) was scored as negative.

The results of the dipstick tests performed in Santa Luzia were not included, because in a high proportion of the dipsticks positive control band staining was not observed. In the other 8 municipalities, all tests performed showed staining of the positive control band[3 6 7].

Blood elution

The blood was eluted the day before testing in ELISA. Two 3.17 mm-diameter discs were punched from the blood-impregnated paper and eluted overnight in 50µl of phosphate buffer saline containing 0.1% (v/v) Tween 20 (PBST). The two paper discs contained approximately 5µl blood, corresponding to 2,5µl serum. The following day, a final serum dilution of 1:100 was obtained by adding 200µl of PBST containing 10% (v/v) normal goat serum (NGS) at least one hour prior to use in ELISA plates.

ELISA

ELISA for the detection of IgM antibodies to PGL-I of *Mycobacterium leprae* was performed essentially as previously described[4]. ND-O-BSA was used as the semi-synthetic analogue of PGL-I. This antigen was diluted in carbonate buffer (pH 9.6) in a sugar concentration of 0.023µg/ml. As a control, 0.1µg/ml bovine serum albumin (BSA) was used. Nunc-Immunoplates-II (Life Technologies, Taastrup, Denmark) plates were coated with 50µl/well of antigen or control. The plates were incubated overnight at 37°C in a moist chamber. Microtiter plates were blocked for 60 min with 100µl of 1% (w/v) BSA in PBS. Next, 50µl of the eluted blood was added to each well. This was incubated at 37°C for 60 min. After incubation, the plates were washed 4 times with PBS containing 0.1% (v/v) Tween-20 (PBST). Peroxidase conjugated anti-human IgM conjugate (Capple/Organon Teknika, Turnhout, Belgium) was added (50µl/well) at a 1:2000 dilution in PBST-10% NGS. After incubation at 37°C for 60 min, the washing procedure was repeated and 50µl of the Sigma 3,3',5,5'- tetramethyl-benzidine (TMB) liquid substrate system was added to each well. In order to control for plate-to-plate and day-to-day variation, a positive reference serum was included in quadruplicate on each plate. The color reactions of the entire plate were stopped with 50µl 2.5N H_2SO_4 when the optical density (OD) at 450nm of the reference control serum reached 0.6. All sera were tested in duplicate and the ELISA results were expressed as mean absorbance of the duplicates. The final OD value of each serum sample was calculated by subtracting the OD value of wells coated only with BSA from the OD value of the test wells coated with ND-O-BSA. The cut-off value used for positivity was OD=0.150; any criterion for positivity is arbitrary since the distribution of antibody concentration is unimodal[10].

Data analysis

The data were analyzed using Epi-info version 6.04b and the Excel software package. Analyses of variance were applied as indicated in the text. All probabilities presented are two-tailed. The heterogeneity of seroprevalence within the different clusters was examined with a goodness-of-fit test ($\Sigma\ [(O - E)^2/E]$).

Kappa values express the agreement beyond chance. Generally, a kappa value of 0.60 to 0.80 represents a substantial agreement beyond chance and a kappa value of >0.80 represents almost perfect agreement beyond chance[11].

RESULTS

Geographic and demographic characteristics

Population size, area and population densities of the municipalities are presented in **Table 1**. Expressive variation occurred between municipalities, such that the concentration of inhabitants ranged from 29 to 672 persons per km².

TABLE 1

Geographic and demographic characteristics per municipality (1998).

	Population	Area (km²)	Population density (people/km²)
Minas Gerais			
Governador Valadares	231,421	2,349	99
Santa Luzia	157,926	235	672
Barbacena	109,769	758	145
Espirito Santo			
Aracruz	61,339	1,427	43
Colatina	105,591	1,799	59
Santa Teresa	19,669	687	29
Santa Catarina			
Itajaí	130,777	303	432
Laguna	47,839	353	136
Tubarão	86,936	284	306

Leprosy indicators

The leprosy detection rates for the 5 years preceding the study are presented in **Table 2**.

Assessment of the data from the different municipalities showed that the leprosy detection rate was relatively stable over the preceding 5 years in most municipalities, except for Santa Teresa (ES), which showed an abrupt increase in the case detection rate in 1998. Governador Valadares (MG) was the municipality that presented the highest case detection rate, with a mean of 11.2/10,000. Aracruz (ES) and Colatina (ES) presented similar, medium detection rates of 3.5-3.7/10,000 in the preceding 5 years. Santa Luzia (MG), Barbacena (MG), Itajai (SC), Laguna (SC) and Tubarão (SC) with mean case detection rates below 1/10,000 were considered low-endemic municipalities.

TABLE 2

Leprosy detection rate per municipality.

	Detection rate/10,000					
	1995	1996	1997	1998	1999	Mean*
Minas Gerais						
Governador Valadares	14.8	7.7	11.8	10.9	10.6	11.2
Santa Luzia	1.3	0.9	0.6	0.9	1.2	1.0
Barbacena	0.0	0.3	0.2	0.3	0.0	0.2
Espirito Santo						
Arcruz	3.0	3.8	4.4	4.8	2.3	3.7
Colatina	4.1	2.9	4.3	3.4	3.0	3.5
Santa Teresa	1.3	1.0	1.0	4.1	3.6	2.2
Santa Catarina						
Itajaí	1.6	1.5	0.4	0.8	0.3	0.9
Laguna	0.9	0.2	0.5	0.2	0.2	0.5
Tubarão	0.8	0.1	0.2	0.2	0.6	0.5

*mean detection rate 1995-1999.

Study population

The characteristics of the study population are shown in **Table 3**. A total of 162 schools were selected from all those registered and within these, 224 clusters were studied.

TABLE 3

Characteristics of the study population per municipality.

	Schools			Pupils					
	nº public	nº private	nº clusters	% participation[1]	nº registered[2]	nº studied	% M/F	Mean age	% BCG[3]
MG									
Gov. Valadares	28	0	30	45	6,779	843	50/50	12.1	99.9
Santa Luzia	19	0	25	51	4,871	787	46/54	12.1	99.6
Barbacena	12	0	25	51	2,304	755	43/57	11.8	100
ES									
Aracruz	12	3	26	84	2,612	815	49/51	11.4	97.9
Colatina	17	3	25	91	2,707	794	47/53	11.3	98.5
Santa Teresa	6	1	18	70	613	432	54/46	11.5	93.8
SC									
Itajaí	21	4	25	60	1,789	1,014	48/52	11.7	98.9
Laguna	12	2	25	60	1,222	738	52/48	12.0	95.4
Tubarão	21	2	25	39	2,292	895	44/56	11.7	98.3

BCG: bacillus Calmette-Guérin, MG: Minas Gerais State, ES: Espírito Santo State, SC: Santa Catarina State, M: male, F: female.

[1]invited pupils that parents allowed to participate in the studied.

[2]total number of children in the 5th grade class.

[3]BCG vaccinated children.

The participation of the children varied significantly between the municipalities, depending on the permission given by the parents, such that participation ranged from 45% to 91% (**Table 3**).

The ratio males/females within municipalities varied from 43/57 to 54/46. The mean age varied from 11.3 to 12.1 years-old. BCG vaccination coverage was almost 100% in all municipalities. A significant difference was observed in the study population between municipalities with regard to sex (Chi square = 27.1, $p < 0.001$), age (F Statistic = 33.2, $p < 0.00001$) and BCG status (Chi square = 128.2, $p < 0.00000001$).

Serology

Table 4 shows the results of the serological examination by ELISA and the ML Dipstick. The overall agreement between ELISA and the ML Dipstick was 90.2%, kappa value 0.6. The seropositivity results ranged from 12% to 25% for ELISA and 8.5% to 14.4% for ML Dipstick. No correlation between leprosy detection rate and seropositivity rates for both ELISA and the ML Dipstick was established. **Figures 1** and **2** show the percentage of seropositivity by ML Dipstick and ELISA, respectively, in relation to the leprosy detection rates.

TABLE 4

Seropositivity rates among school children per municipality.

	ELISA (%)			ML Dipstick (%)		
	seroprevalence	LL	UL	seroprevalence	LL	UL
Minas Gerais						
Governador Valadares	18.6	16.2	21.1	14.2	10.7	17.7
Santa Luzia	23.0	18.9	26.8	-	-	-
Barbacena	15.0	10.5	15.2	12.8	12.4	18.1
Espirito Santo						
Aracruz	24.0	22.8	25.3	14.4	11.2	17.5
Colatina	14.0	11.8	15.6	10.1	7.3	12.8
Santa Teresa	15.0	11.9	18.2	17.4	14.8	19.9
Santa Catarina						
Itajaí	16.9	14.7	19.0	14.2	12.5	15.9
Laguna	12.0	9.6	13.7	12.5	10.4	14.6
Tubarão	12.0	9.8	14.1	8.5	7.0	10.0

LL: lower limit, 95% confidence intervals.

UL: upper limit, 95% confidence intervals.

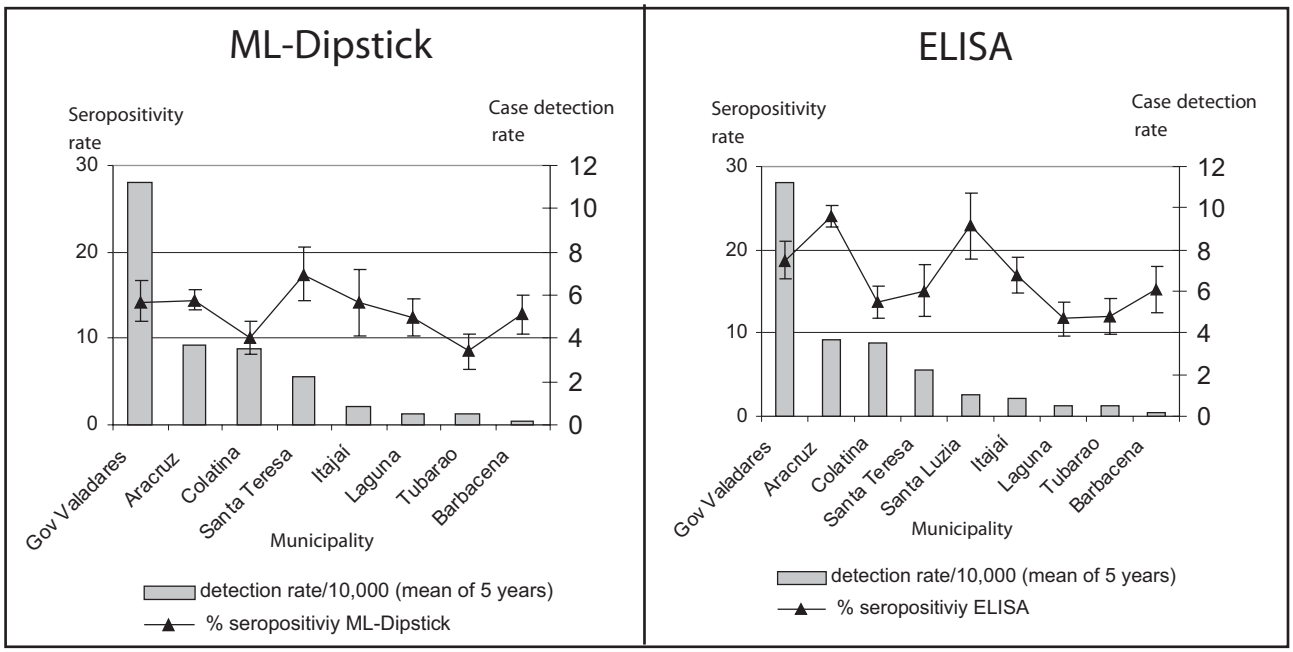

FIGURE 1

Seropositivity and case-detection per municipalities.

To investigate how dipstick seropositivity rates were distributed among different clusters and, consequently, in different schools, the expected seropositivity rates were calculated and compared with the observed seropositivity rates. Of the 145 schools studied with dipstick in 8 municipalities, 20 (13.8%) showed a significantly different seropositivity from the mean seropositivity observed in the municipality. In total, 8 (5.5%) schools had a significantly lower seropositivity than expected. Among private schools, significantly more schools showed a lower seropositivity (5/15) than expected compared to public schools (3/130) (Chi square = 19.2, p< 0.002). **Figure 2** shows the percentage of seropositivity by ELISA per school type.

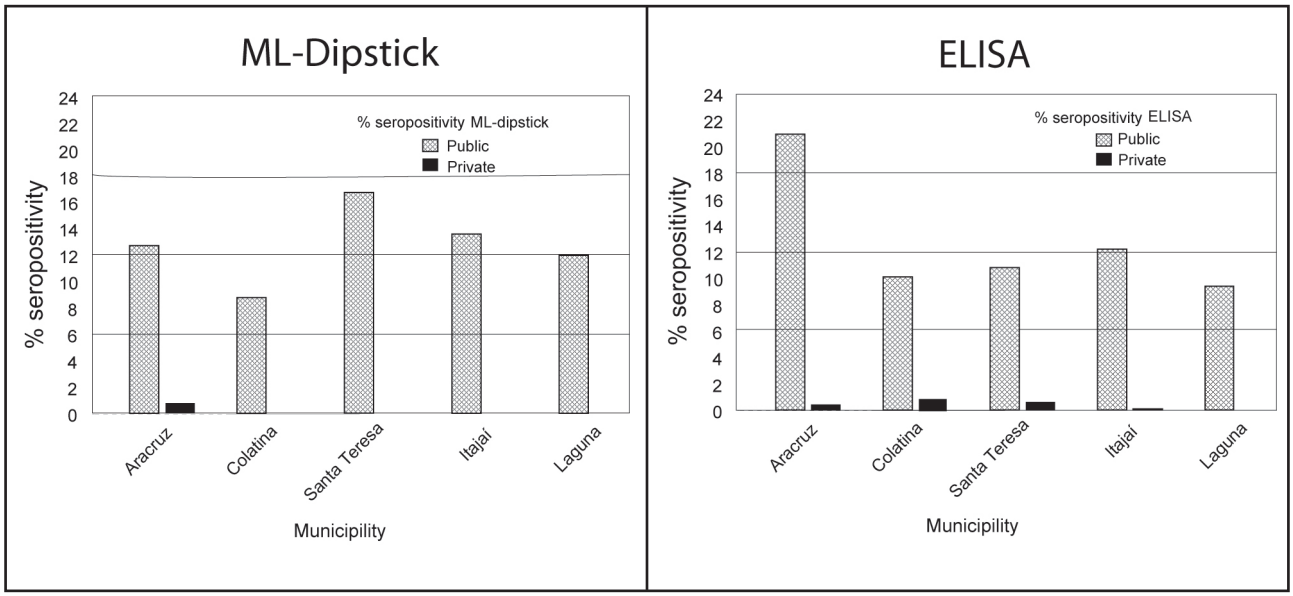

FIGURE 2

Seropositivity in municipalities where public and private schools were studied.

DISCUSSION

Leprosy control programs would benefit from an easy method to estimate disease prevalence and to assess the effect of leprosy control measures on the prevalence of the disease. This would help such programs monitor and maximize the effect of their interventions. In a leprosy-endemic area in Indonesia, it was shown that the seropositivity rates of antibodies to PGL-I by surveys in schools might be a useful indicator of leprosy prevalence at the district level[16]. A strong correlation was found, but only two levels of endemicity were studied. In the present study, more levels of endemicity were included and a methodology that could be easily used in the field was applied.

The study included clusters of school children from 9 municipalities in 3 states in Brazil with differing leprosy detection rates. Seropositivity was studied using both ELISA and the ML Dipstick and the results were compared with the reported leprosy detection rates. The present results show a widely varying distribution of seropositivity in the communities independent of the number of leprosy cases detected. These results are in disagreement with the survey of school children in Indonesia[16]. Seropositivity was not capable of differentiating between different levels of leprosy endemicity and no relation was found between seropositivity and leprosy indicators. Furthermore, no clear differences in the patterns of seropositivity between ELISA and the dipstick were observed.

Assuming that seropositivity is an indicator of infection, there may be a number of explanations why this study obtained data indicating that seropositivity was widely distributed in the community:

i) *Mycobacterium leprae* subclinical infection may be widespread, but the immunity of the population prevents leprosy from becoming apparent;

ii) *Mycobacterium leprae* may be transmitted not only by contact with clinical patients, but also by contact with subclinically infected individuals, by *M.leprae* present in the environment[11] or even by a reservoir host as yet unidentified; or

iii) infection in the community by environmental (myco)bacteria and/or common infections may result in a cross-reactive anti-PGL-I antibody response.

In addition to these more general factors, there are a number of other factors related to the study design and execution of the study that could possibly explain why no clear relation was observed between leprosy indicators and serology.

First, it is possible that the reported detection rates were not always accurate. The selection of municipalities with different leprosy incidence rates was based on the data and experience of the national leprosy control program. Only municipalities with stable leprosy control programs during the preceding 5 years were included; however, the possibility that in some areas the number of hidden leprosy cases could be higher than expected can not

be excluded. Leboeuf and Grossi estimated that only 59 to 84% of all leprosy cases were detected in Minas Gerais State[14]. This could explain the higher than expected seropositivity rates found in Santa Lucia and Barbacena, but not in Governador Valadares, where the seropositivity rate was lower than expected.

Second, the possibility that, in some municipalities, the study population was not representative for the total population also cannot be ruled out. The study design using cluster sampling in a survey of school children should have been appropriate and, in principle, the sample size was large enough to represent the community. However, participation of the children was dependent on the consent of their parents and their presence at school at the time of the study. These factors could have introduced bias. It is possible that children who had household contact were prevented from participating by their parents because of the stigma associated with leprosy, especially in high endemic areas. Socioeconomic factors could be another reason for not participating in the study. During the fieldwork in the State of Espirito Santo, many children missed school because they needed to help with harvesting the coffee beans. Indeed, the highest leprosy endemic municipality (Governador Valadares) showed a low participation rate.

Third, the antigen used for determining seropositivity in the Indonesia study was the semi-synthetic natural trisaccharide bound to bovine serum albumin through a phenyl linker (NT-P-BSA), which, in addition, was used in an agglutination test. In this study a similar antigen was used, a semi-synthetic natural disaccharide bound to bovine serum albumin through an octyl linker (ND-O-BSA). A good correlation between the two antigens was reported ($r = 0.81$), though the specificity of NT-P-BSA is higher (95.4%) than ND-O-BSA (93.1%)[7]. The use of antigens with different specificity could impair the comparison of results from other studies. Alternatively, the use of whole blood instead of serum may have played a role.

When analyzing the 20 clusters for which seropositivity was lower or higher than expected, a significant difference was observed between private schools and public schools.

Private schools were more likely to present a lower seropositivity. This difference was statistically significant and is in agreement with the hypothesis that seropositivity is a reflection of exposure to *Mycobacterium leprae* and is likely to be related to socioeconomic status. Differences in the socioeconomic status of the pupils attending public and private schools could be the explanation for the difference in infection distribution obtained. On the other hand, the higher seropositivity observed in public schools could well be the result of cross-reactivity. Exposure to other environmental mycobacteria and therefore higher than expected seropositivity rates, could just be a reflection of non-specific binding of cross-reacting antibodies to the antigen.

Based on the results obtained, it is not possible to make definitive conclusions concerning the hypothesis that correlation occurs between seropositivity and leprosy prevalence in a community.

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