

Study of the Antibody Response against *Mycobacterium tuberculosis* Antigens in Warao Amerindian Children in Venezuela

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This study was aimed at investigating alternate methods for serodiagnosis of tuberculosis (TB), which are needed because bacteriologic diagnosis of childhood TB is difficult. A selection of 80 serum and saliva samples were tested from Warao indigenous children under 15 years of age; 34 high TB suspects (28 positive and 6 negative for the tuberculin skin test, TST) and 46 healthy contact children (32 positive and 14 negative for the TST). Several enzyme-linked immunosorbent assay (ELISA) serological tests were developed to test for Mycobacterium tuberculosis-specific antibodies, including serum IgA, IgG, IgE, and secretory IgA (sIgA) in saliva against 3 specific antigens (PPD, HSP60, 38 kDa). Of these, 2 antigens, PPD and 38 kDa, showed significantly higher reactivity. The sensitivity and specificity of these tests for diagnosis remained limited, between 26.5% and 38.2%, and 77.4% and 97%, respectively. Of all the samples studied and combinations realized between all isotypes and antigens combined with 3 isotypes (anti-PPD IgG, IgE, and anti-38kDa sIgA) managed to detect the largest number of patients, showing an improved sensitivity level of 64.7%, although specificity levels dropped to 81.8%. These results were compared with the Omega diagnostics commercial kit results. The commercial kits showed significantly lower reactivity (sensitivity of 20% and 13.33% to Myco G and Complex Plus, respectively) and a specificity of 100%. This study shows that in indigenous populations of Venezuela, where invasive procedures cannot be used to select samples but evaluation with a chest X-ray for radiological studies is available, the combination of 3 specific isotypes may be a useful tool to increase diagnostic accuracy with pulmonary TB in this population, when used together with clinical and epidemiological criteria.

Key words: tuberculosis (TB) - Warao Amerindians - tuberculin skin test (TST) - IgA - IgG - IgE - secretory IgA (sIgA)

It has been reported that tuberculosis (TB) is a public health problem among indigenous communities of Venezuela, of whom 95% live in rural areas (OCEI 1992). Venezuelan Health Services reported among non-indigenous populations a rate between 26.1 and 24.8 and among indigenous populations a rate between 155.6 and 129.4 per 100,000 inhabitants between 1997 and 2001 (MSDS 2002). Since 1999, Delta Amacuro State has presented the highest rates, between 93.2 and 81 per 100,000 inhabitants. Of these, 90% of the cases were present in the indigenous Warao population, with a very high prevalence of adult TB (MSAS 1999, MSDS 2002). When we carried out a TB diagnostic study on the Warao child population, we found the extremely high TB rate of 3190/100,000 in children under 15 years of age (Fernández de Larrea et al. 2002).

Studies that address the difficulties of TB diagnosis in children and attempts to improve diagnostic methods are not new (Eamranond & Jaramillo 2001). Although there are various scoring systems for the diagnosis of TB in children, these are often underutilized, misused or not used at all, and the sensitivity and specificity are variable. Since the bacilli-containing sputum is usually swallowed, especially when the children cannot produce a sufficient sputum sample, several diagnostic methods – including the polymerase chain reaction (PCR), high resolution computerized tomography (CT) and improved serological tests – are recommended (Eamranond & Jaramillo 2001, Honore et al. 2001).

In recent years, antigen or antibody detection tests such as enzyme immunoassays have largely replaced culture for the diagnosis of many infectious diseases because they can be performed in intermediate-level laboratories with relatively simple equipment (Mabey et al. 2004). In relation to the serological tests for immunodiagnosis of TB, several combinations using different antigens or specific antibodies to increase sensitivity and specificity of serological testing of TB in adult patients have been reported (Daniel & Debane 1987, Chiang et al. 1997, Pottumarthy et al. 2000).

Very few studies have addressed the problem in the diagnosis of childhood TB. The use of ELISA for

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immunodiagnosis of TB has shown that the humoral response to mycobacterial antigens is low in children, especially when combined responses against antigens or specific antibodies are not used (Starke 1993, Swaminathan et al. 1999, Sant'Anna et al. 1999). Non-recombinant and recombinant antigens of *Mycobacterium tuberculosis* such as purified protein derivative (PPD) and 38 kDa and HSP60, respectively have been used for the serodiagnosis of TB. There are many reports on the sensitivity and specificity of these antigens for serodiagnosis of TB, with the results being variable (Radin et al. 1983, Young et al. 1986, Alde et al. 1989, Zheng et al. 1994, Chiang et al. 1997, Raja et al. 2002).

Since in rural areas of Venezuela such as those of the indigenous Warao communities, where there is no access to a large hospital, and considering that invasive procedures cannot be used to take samples in these communities, in the absence of an ideal standard test, a positive result from a more sensitive, less specific test is considered a reliable indicator of children highly suspected of TB. It is essential to improve the diagnosis of TB in this childhood population. In this paper we report on the evaluation of several ELISA serological tests that were used to detect *M. tuberculosis*-specific antibodies, serum IgA, IgG, and IgE against PPD and HSP60 and secretory IgA in saliva against 38 kDa antigens.

MATERIALS AND METHODS

Since 1996 the Regional Delta Amacuro Tuberculosis Program and the Tuberculosis Laboratory of the Institute of Biomedicine have been actively diagnosing TB cases among the Warao communities based on respiratory symptoms characteristic of TB, the tuberculin skin test (TST), and smears and/or cultures; they have been prescribing specific treatments. A descriptive transversal study was carried out between 2000 and 2001 in which a total population of 502 children under the age of 15 years old residing in the visited communities was examined. A total of 80 children were selected for the study. A single serum sample and saliva sample were collected from all children studied (of either sex and with average age of 8.24 ± 4.84 years old). Bearing in mind that the WHO uses the criteria of presence of weight loss or inadequate weight gain, coughing or wheezing and a history of household contact with an adult case of lung TB, and that these criteria have had a positive predictable value between 63% and 70% (Migliori et al. 1992, Houwert et al. 1998), we adopted a previously-reported scheme (Fernández de Larrea et al. 2002), which takes into account the clinical, nutritional, and epidemiological factors in the children, specifically (1) clinical and nutritional criteria and positive reactivity to tuberculin, and (2) clinical and nutritional criteria, with negative tuberculin and positive household contact. During our study specific attention was given to Warao children with respiratory symptoms which suggested TB and were therefore further evaluated with a chest X-ray. Radiological studies suggested that 34 children were highly suspected TB cases. The children were grouped as follows: Warao patient group – children with probable active TB [clinically, radiologically and/or bacteriologically diagnosed (Fernández de Larrea et al. 2002)] before treat-

ment ($n = 34$, 15 females and 19 males), 28 positive and 6 negative for the TST; Warao control group – healthy contact controls ($n = 46$, 31 females and 15 males), 32 positive and 14 negative for the TST. All control children were evaluated with the rating system mentioned above and none was found with characteristic signs of TB. Registration of the presence or non presence of BCG scars was carried out.

Inclusion criteria for both study groups - (1) Patients and controls included a Warao population of children between 1 month and 15 years old without or with respiratory symptoms, the latter suggesting pulmonary tuberculosis; (2) Only the O blood group Rh+ children of both patient and control groups were included in this study because the Venezuelan indigenous populations are fundamentally O blood group (Arends 1992); (3) Since in Venezuela's National Tuberculosis Program the BCG vaccine is given in the first month of age, patients and controls included children with presence or non presence of BCG scars; (4) Patient group: children with probable active TB, based on clinical diagnosis, thorax radiography, smears stained with Ziehl-Neelsen and/or microbiological culture without or with positive TST (≥ 10 mm); (5) Control group: healthy subjects with no evidence of clinical, microbiological or radiographic signs of pulmonary TB without or with positive TST; (6) Only patients with probable active TB who were HIV negative were included in this study, (the results revealed no case of HIV-positive TB among the Warao children). Consent was obtained from all participants or their legal representatives (children or parents, respectively), who signed a "consent form" agreement before blood and saliva samples were selected. The approval of the Ethical Commission of the Biomedicine Institute was also obtained.

Confirmatory studies

Radiological - Despite difficult access, thorax radiology studies were performed on all highly suspected cases according to standard techniques in post-anterior projection. This took place in the radiology service of the Hospital Luis Razzetti, Tucupita.

Bacteriological - Given that invasive procedures cannot be used to select samples in these communities, a study of secretions of the pharynx and attempts to obtain samples of sputum by expectoration in children older than 10 years old was carried out in all highly suspected cases. Smears from sputum were stained by the Ziehl-Neelsen direct method. For each specimen (sputum or secretions of the pharynx) 2 tubes of modified Ogawa egg medium and Lowenstein-Jensen were inoculated using the swab method of Kudoh and Kudoh (for sputum as well as ooze) (Kudoh & Kudoh 1974). Specific treatments were initiated in all newly identified cases of TB following the norms of the Venezuelan National Tuberculosis Control Program (MSAS 1996).

Therapeutic conduct - Anti-TB treatment was initiated on all highly suspected cases, where radiological evidence suggestive of TB or bacteriological confirmation by bacilloscopy or culture was found. During and after anti-TB drugs treatment, clinical and nutritional monitoring on all highly suspected patients was carried out in

order to evaluate the improvement of these aspects as therapeutic confirmation, which allowed us to corroborate the diagnosis.

Immunological studies

The TST - The TSTs were performed on all the individuals of this study using 2 tuberculin units of PPD of *M. tuberculosis*, strain RT-23, supplied by the Copenhagen World Health Organization reference laboratory (Denmark) and in current use by the Venezuelan Health Services. Testing and reading were done according to international guidelines (Snider 1982, Arnadottir et al. 1996). In order to evaluate the possibility of non-specific anergy, skin test reactivity to *Candida albicans* was also carried out. The *Candida* antigen was provided by the Laboratory of Micology, Institute of Biomedicine. Both intradermal injections of 0.1 ml solutions were administered by a trained nurse (the Regional Delta Amacuro Tuberculosis Program) into the volar surface of the left (tuberculin PPD) and right (*C. albicans* antigen) forearm. The diameters of indurations were measured 72 h after inoculations; indurations of ≥ 10 mm and ≥ 5 mm were used as the criterion for infection with *M. tuberculosis* and *C. albicans*, respectively.

Antibodies to M. tuberculosis antigens - Immuno-enzymatic assays were performed blindly by a lab technician, and developed and standardized in our laboratory for the measurement of antibodies (IgA, IgG, IgE, sIgA) against PPD, HSP60, and 38 kDa antigens. Each assay included positive and negative sera and blanks to control non-specific binding.

Serum IgA anti-PPD - The levels of anti-PPD IgA were determined in serum by an ELISA as follows. Sera were isolated from venous blood obtained from controls and TB patients. Microtiter plates (Immunolon) with 96 wells were coated with PPD (Statens Seruminstitut, Copenhagen), (1 μ g/well of each antigen in carbonate-bicarbonate buffer pH 9.6) overnight at 4°C. Excess protein binding sites were blocked by incubation with 1% BSA in PBS at 37°C for 2 h. Then the plates were washed 4 times with PBS containing 0.1% Tween 20. Samples diluted 1:50 in PBS containing 0.5% BSA were added and plates were incubated for 2 h at 37°C. The plates were washed 4 times, then incubated with the second antibody (peroxidase-conjugated monoclonal antibody anti-alpha chain IgA (Sigma-Aldrich, US) diluted 1:1000 in blocking solution) for 1 h at 37°C and washed 4 times. After washing, substrate solution (30 μ l of 30% H₂O₂ and 10 mg o-phenylenediamine (OPD) dihydrochloride, Sigma-Aldrich) in 25 ml citrate buffer, pH 5 was added and incubated for 6 min at room temperature. Colour development was measured in an ELISA reader at 492 nm.

Serum IgG anti-PPD and HSP60 - The levels of anti-PPD and HSP60 IgG were determined in serum by an ELISA similar to that described for anti-PPD serum IgA. The serum samples were diluted 1:400 in blocking solution, and peroxidase-labeled anti-gamma chain IgG monoclonal antibody (Anti-human IgG HRP-conjugate, Promega Corporation, US) was used as the second antibody.

Serum IgE anti-PPD - The levels of anti-PPD IgE were determined by a similar ELISA to that described for anti-PPD IgA. The serum samples were diluted 1:50 in block-

ing solution, and biotin-labeled anti-epsilon chain IgE monoclonal antibody (Anti-human IgE, Epsilon Chain Specific, Vector Laboratories) was used as the second antibody.

sIgA anti 38 kDa - The levels of sIgA in saliva were determined by a similar ELISA to that described for serum IgA anti-PPD. Microtiter plates (Immunolon) were coated overnight at 4°C with 38 kDa antigen (1 μ g/well of antigen in carbonate-bicarbonate buffer pH 9.6). The saliva samples were diluted 1:10, and peroxidase-conjugated monoclonal antibody anti-alpha chain IgA (Sigma-Aldrich) was used as the second antibody. A group of 38 healthy non Warao children negative for the TST from a non endemic area of Caracas was included for the evaluation of positive sera based on the cut-off values.

Commercial kits - The results were compared with the Omega diagnostics commercial kit results (Pathozyme-Myc G[®], Omega Diagnostics, and Pathozyme-TB Complex Plus[®], Omega Diagnostics).

Statistical analysis - The evaluation of positive sera was based on a positive score represented by optical density values (OD) above the cut-off point of the mean value plus 2 standard deviations of sera from a healthy control group from Caracas, the capital of Venezuela. The results are shown in OD x 1000. The statistical analysis used to compare the significance of the differences between the percentage values of sIgA and serum IgA, IgG, and IgE levels was Fisher's exact test and the Pearson test for correlations. The significance of the differences among the mean of the levels of isotypes between patient and control groups was estimated by Student's *t* test.

RESULTS

Clinical and bacteriological studies - Radiological: thorax radiology was practiced on all highly suspected cases, which comprised 34/80 (42.5%) of the children. Bacteriological: study of 22 secretions of the pharynx and 5 sputum samples obtained by expectoration in children older than 10 years old showed that in 3/34 (8.8%) of patients there was bacteriological confirmation, while 1 sputum (20%) and 1 secretion of the pharynx (4.5%) were positive by culture and 1 sputum by smear examination (20%).

Immunological responses for the tuberculin PPD skin reaction - Distribution of indurated tuberculin PPD responses is shown in Fig. 1. Reactions of ≥ 10 mm were found in 28/34 (82.3%) of the patients and in 32/46 (69.5%) of the controls (data not shown). Tuberculin negative reactivity of controls and patients was found in 14/46 (30.4%) and 6/34 (17.6%), respectively. In the latter group, we found that 14.7% of children presented reactions of 0-4 mm and 2.9% of 5-9 mm. When the skin test reactivity was carried out in order to evaluate the state of non-specific anergy in patients negative for the TST, we found that there was a high percentage of patients negative for tuberculin and *Candida* skin tests (80%). A significant correlation between patients with tuberculin and *Candida* negative responses was found ($p < 0.001$) (data not shown). Positive reactions of patients and controls showed a similar frequency of reactivity (26.4% and 23.9% 10-14 mm, respectively and 55.8% and 45.6% ≥ 15 mm, respectively) (Fig.

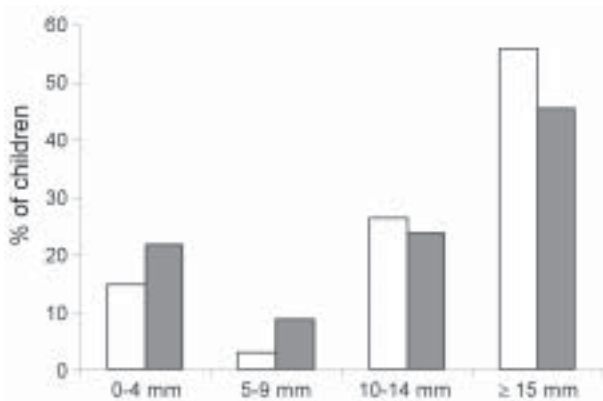


Fig. 1: distribution of the response to the tuberculin skin test (TST response) among the population of children studied. Patients (n = 34) (empty bars) and controls (n = 46) (filled bars)

1). The results concerning the cellular immune response to TST and the BCG vaccination status showed an increased percentage of reactions ≥ 10 mm in patient and control children older than 7 years old [15/28 (53.5%) and 22/32 (68.7%), respectively] independent of the BCG status (data not shown).

Antibodies to specific antigens according to the TST

- The mean and distribution values of the OD related to the cut-off are shown in Fig. 2. The cut-off criteria for anti-PPD IgA, IgG, and IgE and anti-38 kDa sIgA were 0.384 (Fig. 2A), 0.674 (Fig. 2B), 0.633 (Fig. 2C) and 0.730 (Fig. 2D), respectively, of patients and controls according to the TST. The percentages of patients and controls with positive or negative responses to specific antigens according to the TST are shown in Fig. 3. For anti-PPD IgA, concerning the IgA specific response according to the TST, sera from patients and controls positive or negative for the TST showed a similar percentage of subjects with IgA antibodies reactive to PPD antigen. Similarly, there was no statistically significant difference in the calculation of the percentage of children with anti-HSP60 IgG response between children positive and negative for the TST (data not shown). In contrast, concerning the anti-PPD IgG response, sera from patients positive for the TST showed a significant percentage of children with specific IgG (32.1%, 9/28) in comparison to controls positive for the TST (3.1%, 1/32) $p < 0.004$ (Fig. 3).

Among children negative for the TST in the patient group, there was a significant percentage of subjects with anti-PPD IgE (66.6%, 4/6) in comparison to patients positive for the TST (21.4%, 6/28), $p < 0.04$ (Fig. 3). In the

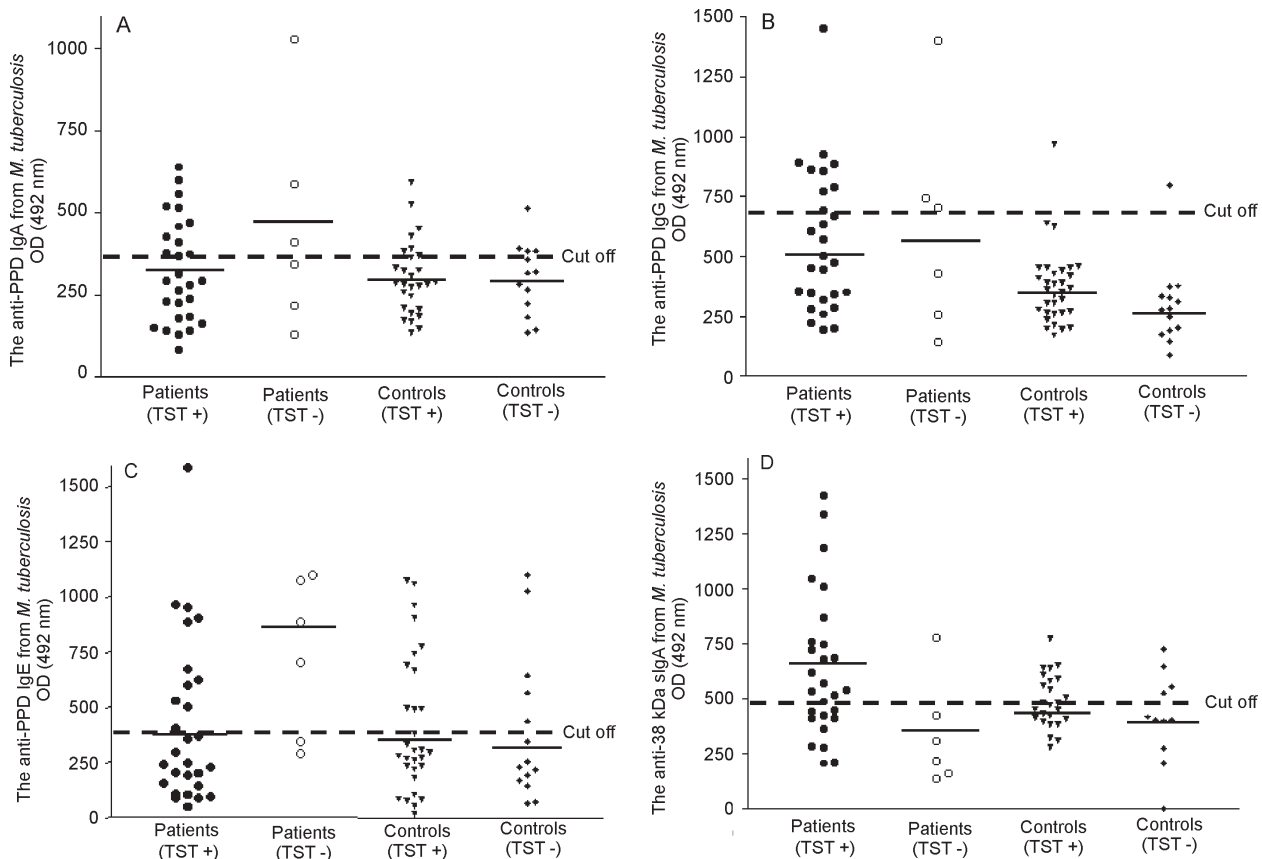


Fig. 2: distribution values of optical density (OD) and cut-off of the antibody tests. Patients and controls humoral reactivity against tuberculin PPD and 38 kDa antigens, expressed as OD. The horizontal line and horizontal bar indicate the mean and cut-off, respectively. A group of 38 healthy non Warao children negative for the tuberculin skin test (TST) from a non endemic area of Caracas was included for the evaluation of positive sera based on the cut-off value. The cut off for anti-PPD IgA, IgG, IgE, and anti-38 kDa sIgA were 0.384 (A), 0.674 (B), 0.633 (C), and 0.730 (D), respectively.

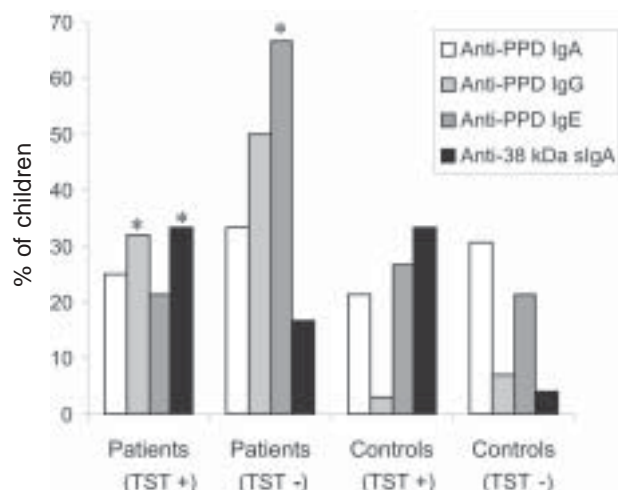


Fig. 3: data representing the percentage of patients and controls children with specific antibodies according to the response to the tuberculin skin test (TST) response. Anti-PPD IgA (□), anti-PPD IgG (▤), anti-PPD IgE (▥), anti-38 kDa sIgA (■). The evaluation of positive sera was based on a positive score represented by optical density (OD) values above the cut-off point of the mean value plus 2 standard deviations of sera from a healthy control group (n = 38) from Caracas, the capital of Venezuela. For anti-PPD IgG and anti-38 kDa sIgA significant differences were observed between patient (TST +) and control (TST +). For anti-PPD IgE a significant difference was observed between patient (TST -) and patient (TST +).

control group, there was no statistically significant difference in the percentage of children with specific IgE response between children positive and negative for the TST - 26.6%, 80/30 and 21.4%, 3/14, respectively (Fig. 3). Patients positive for the TST comprised a significant percentage of children with sIgA antibodies reactive to 38 kDa (33.3%, 9/27) in comparison to controls negative for the TST (4%, 1/25), $p < 0.01$ (Fig. 3).

Sensitivity and specificity of the tests - The sensitivity and specificity of the different tests are shown in the Table. The specific antibodies anti-38 kDa sIgA, anti-HSP60 IgG, anti-PPD IgA, IgG, and IgE showed a sensitivity between 26.5% and 38.2% and a specificity between 77.4% and 97%. The combination of specific serum antibodies IgG and IgE against PPD and sIgA in saliva to 38 kDa showed a sensitivity of 64.7% and a specificity of 81.8% (Table). These results were compared with the Omega diagnostics commercial kit results (Pathozyme Myco G[®] and Pathozyme Complex Plus[®]). The commercial kits showed significantly lower reactivity (sensitivity of 20% and 13.33% to Myco G and Complex Plus, respectively) with 100% specificity.

DISCUSSION

Bearing in mind that the sensitivities of culture and smear drop precipitously when applied to children, and other diagnostic test/criteria take on greater importance such as serological tests, especially combined tests (Alifano et al. 1996, Gupta et al. 1997, Swaminathan et al. 1999, Pottumarthy et al. 2000, Imaz et al. 2001), the present

TABLE
Sensitivity and specificity of the different tests

Specific antibodies	Sensitivity (%)	Specificity (%)
Anti-PPD IgG	38.2	95.8
Anti-PPD IgE	32.4	77.4
Anti-PPD IgA	26.5	92.8
Anti-38 kDa sIgA	36.1	91.6
Anti-HSP60 IgG	33.3	97.0
MTB	20.0	100.0
M. sp.	13.3	100.0
Anti-PPD IgG, IgE and Anti-38 kDa sIgA	64.7	81.8

PPD and 38 kDa: *Mycobacterium tuberculosis* antigens; MTB: Panthozyme Myco G[®], M. sp.: Panthozyme Complex Plus[®]

study was performed to evaluate the diagnostic potential for detection of IgA, IgG, IgE, and sIgA against *M. tuberculosis* antigens. The results showed that in a significant percentage of children, the antibody levels to different *M. tuberculosis* antigens were raised in patients highly suspected of TB and were particularly high for anti-PPD IgE in patients with tuberculin negative reactivity.

The sensitivity of the anti-HSP60 IgG, and anti-PPD IgG, IgA, and IgE and anti-38 kDa sIgA tests for immunodiagnosis remained limited between 26.5% and 38.2%, which makes it a poor diagnostic tool for disease confirmation. In order to improve the sensitivity tests, combinations realized between 3 isotypes and 2 antigens – anti-PPD IgG, IgE, and anti-38kDa sIgA – used to detect the larger number of patients, allowed us to obtain a sensitivity level of 64.7%, even though specificity levels dropped to 81.8%. When we carried out a study of this combination method in a population of 19 non Warao children, all of them positive for the TST, from a place with low prevalence of latent and active infection (Coporito, Tucupita Municipal District of Delta Amacuro State), the combination showed that the differences of the specificities in these 2 populations were not significant (78.1% and 81.8% in non Warao and Warao children, respectively, data not shown).

As mentioned above 3 antigens were evaluated, of which 2 antigens, PPD and 38 kDa, showed significantly higher reactivity. The PPD, a non-recombinant antigen, had shown variable sensitivity and specificity in previous reports (Radin et al. 1983, Starke 1993). We found for anti PPD-IgG, IgA, and IgE antibodies, a sensitivity between 26.5% and 38.2% and a specificity of 77.4% and 95.8%. Our sensitivity results of 38.2% for anti-PPD IgG are similar to those found by others using PPD (Barrera et al. 1989, Starke 1993, Zheng et al. 1994, Raja et al. 2001). It has been reported that patients with active TB clearly had higher levels of IgG antibody to PPD antigen than did a healthy control group. However, no IgE antibodies were found (Radin et al. 1983). In our work, we found high IgE levels in both control and probable active TB cases. These different findings may be attributable to differences in the respective study population or methodology. Many re-

ports have confirmed that specific IgE antibodies, a marker of Th2 responses, are elevated in TB and were correlated with disease incidence (Yong et al. 1989, Beyer et al. 1998, Adams et al. 1999). In addition, it has been reported that IgE concentrations decreased after successful treatment of TB, presumably due to the enhancement of a Th1 response, which suggests that TB up-regulates the Th2 response (Yong et al. 1989, Beyer et al. 1998). The high percentage of control children with specific IgE antibodies can be explained since the Warao population has a very high prevalence of adult TB, which suggests that Warao children have frequent contact with chronic pulmonary TB (Fernández de Larrea et al. 2002). If true, the high levels of specific IgE could be a result of a recent TB infection, where apparently healthy contact children could have transiently higher antibody levels of this isotype, which could be beneficial from an early diagnosis point of view since children could be identified as individuals susceptible to *M. tuberculosis*. However, false-positive (environmental non-tuberculous micobacteria) does occur.

When the diagnostic for detection of anti-HSP60 IgG was evaluated, our findings showed that both anti-PPD and HSP60 IgG tests presented an overlap of reactivity in the patient group, as has been reported by other researchers (Barrera et al. 1989). These findings suggest that the measurement of the anti-HSP60 IgG antibodies should be excluded from the combination. In addition, it has been proposed by the WHO that the new diagnostic methods should have sensitivity at least higher than bacilloscopy (more than 50%) and specificity higher than 95%. Although our results using anti-PPD and anti-HSP60 IgG showed a higher rate of specificity (95.8% and 97%, respectively), the sensitivity of these tests was lower than 50% (38.2% and 33.3%, respectively). On the other hand, taking into account that the children do not produce a sufficient sputum sample and that among the Warao child population recurrent or overwhelming parasite infections with protozoa and helminthic parasites and also malnutrition at early ages occur (González et al. 2003, Araujo et al. 2003), it seems to be important to improve the sensitivity of any new diagnostic method that puts more emphasis on the early detection of children with TB among high risk groups such as the indigenous Warao (Fernández de Larrea et al. 2002). Since sensitivity of the tests remained limited to between 26.5% and 38.2%, and in order to improve the sensitivity of an antibody detection test, it seems important to combine the responses against different mycobacterial antigens. To assess the applicability of the combination of several antigens and specific antibodies for use in diagnosis of childhood TB, a combination including anti-38 kDa sIgA provided improvement in the diagnosis. There are many reports on the sensitivity and specificity of 38 kDa antigen for serodiagnosis of TB which are about 80% (Young et al. 1986, Jackett et al. 1988). Measurement of 38 kDa antibodies showed a sensitivity greater than 71.4% in adults TB, through a combination of assays for IgG + IgA + IgM, while the specificity was 90% (Uma et al. 2001). In addition, it has been reported that sIgA in sputum was significantly higher in smear negative and culture positive cases compared with culture negative cases (Uma et al. 2001).

Few studies have addressed the detection of sIgA against *M. tuberculosis* antigens in children. In this regard, when we applied the 38 kDa antigen to estimate the specific sIgA in saliva, the measurement of 38 kDa antibodies showed a sensitivity of 36.1%, while the specificity was 91.6%. In the patient group, 16.7% were positive for 38 kDa sIgA but did not have detectable antibodies to PPD (anti-PPD IgG and IgE). On the other hand, our results were compared with the Omega diagnostics commercial kit results (Pathozyme Myco G[®] and Pathozyme Complex Plus[®]). The commercial kits showed significantly lower reactivity (sensitivity of 20% and 13.33% to Myco G and Complex Plus, respectively) with 100% specificity.

Concerning the tuberculin response, it has been reported that overall 10% of patients with active TB fail to react to PPD (McMurray & Echererri 1978, Wright et al. 1995). Our findings showed that a significant 17.6% of tuberculin negative reactivity was found in the patient group. When we used the *Candida* skin test to evaluate the non-specific anergic state in these children, the results showed that 80% of the tuberculous patients who are tuberculin negative did not respond to *Candida* antigen. These findings suggest that there are factors that must be associated with a significant frequency of anergic states in this population, a possibility conditioned by the high rate of parasite, viral and bacterial infections and malnutrition, which are frequently present in these communities. Concerning the antibody response according to the response to the TST, for anti-PPD IgA and IgG and anti-38 kDa sIgA antibodies, there was no statistically significant difference between tuberculin positive and negative children. However, for anti-PPD IgE there was a statistically significant difference between tuberculin positive and negative patients. The latter particularly, in contrast to their diminished lack of skin test reactivity, had high levels of antibodies. The vast majority of these antibodies were found to be of the IgE isotype, generally reported to be interleukin-4-dependent (Yong et al. 1989, Adams et al. 1999), which could condition the high frequency of anergic states observed in this group.

It may seem that the sample size was very small for the evaluation of the different diagnostic tests, but it should be borne in mind that testing is extremely difficult with indigenous communities because of problems of access and ethical considerations. Therefore it is inevitable that sampling is on a reduced scale. These limitations also explain why it was not possible to carry out evaluation of non *Mycobacterium* TB antigens. However, despite the limitations, it should be emphasized that the combination test achieved the highest sensitivity among all the tests, although admittedly the values are still low in terms of general standards. With further analysis it should be possible to improve sensibility and determine positive and negative predictive values. Finally, the present findings suggest that the combination test improves, and allows an increase in, the diagnostic accuracy of pulmonary TB in the Warao childhood population when used together with clinical and epidemiological criteria. In addition to efficient diagnosis, political commitment and public health education at different levels of the government as well as in the Warao population are probably the most important aspects of TB control.

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