Indian Journal of Medical Microbiology, (2002) 20 (3): 145- 149

Original Article

QUALITATIVE AND QUANTITATIVE ANALYSIS OF ANTIBODY RESPONSE IN CHILDHOOD TUBERCULOSIS AGAINST ANTIGENS OF *MYCOBACTERIUM TUBERCULOSIS*

KR Umadevi, B Ramalingam, *A Raja

Abstract

Purpose: Serodiagnosis of tuberculosis in children, using available crude antigens, has been difficult. The tests lack sufficient sensitivity and/or specificity. In this study, western blot analysis of *M. tuberculosis* H37Rv culture filtrate antigen (CFA) was carried out, to identify diagnostically useful antigens. In addition, the CFA was also used in enzyme linked immunosorbent assay (ELISA), to measure antibodies of multiple isotypes. **Methods:** Specific IgG, IgA and IgM antibodies were estimated in the sera from 26 clinically/ bacteriologically diagnosed cases of childhood tuberculosis (CTB) and 61 normal children (CNHS), using culture filtrate antigen. Western blot analysis with culture filtrate antigen was carried out to qualitatively compare the antibody profile among the CTB, with childhood normal controls and adult TB. **Results:** IgG positivity was only 7.6% with culture filtrate antigen in the CTB group, while 3.2% among the controls were also positive. However, the results of IgA and IgM isotypes were better. By combination of all the three isotypes an increased sensitivity of 57.7% with a specificity of 93.5%, was obtained. Immunoblot analysis revealed marked difference among antibodies in the region of 16, 19, 38 and 45kDa between CTB and CNHS. **Conclusions:** Our findings point to a limited sensitivity of 57.7% in ELISA with culture filtrate antigen. However, antibodies around 16, 19,38 and 45kDa region may be useful in differentiating the CTB patients from CNHS by immunoblot assay.

Key words : *Antibody-Isotypes, childhood tuberculosis, ELISA, humoral immune response, Immunoblot*

Tuberculosis of children is an important health problem. According to WHO estimates in 1990, 1.3 million new cases and 450,000 deaths were among the children under 15 years of age.[1] The absolute diagnosis of tuberculosis in children is difficult, because the demonstration of *Mycobacterium tuberculosis* from the secretions and tissues of patients is negative in majority of those affected. Therefore, the diagnosis of childhood tuberculosis is often based on indirect evidences such as peripheral lymphadenopathy, persistent cough or fever, chest radiography and in particular a history of close household contact with an adult with pulmonary tuberculosis and tuberculin testing.

The available skin tests like BCG test and Mantoux test can only predict prior sensitization of an individual to tuberculosis protein through natural infection or vaccination. They do not in any way confirm the diagnosis of active tuberculosis. A negative BCG test can at best be taken as good evidence against the diagnosis whereas a positive test hardly supports the diagnosis of tuberculosis.[2]

Earlier attempts of serodiagnosis, using crude and semipurified antigens, produced by both chemical and immunological methods, have been unsatisfactory in terms of sensitivity and specificity.[3-9] Standardized, purified mycobacterial antigens with species specificity and strong immunogenicity are urgently needed for rapid diagnosis. The present study focuses on, analyzing the antibody response to antigens of *M. tuberculosis*, in order to identify diagnostically useful antigens.

Materials and Methods

Study subjects

The study population consisted of 87 children below the age of 15years. The study population forms a part of a bigger study for evaluation of two ELISA kits, which was conducted at Tuberculosis Research Centre, from July to September 1996[7] and also for flow-cytometry.

Childhood tuberculous patients (CTB) included both pulmonary and extrapulmonary cases (N=26). In addition to clinical examination, radiology and bacteriological tests such as direct smear and culture were carried out. Mantoux test (PPD RT23, 1TU) results

*Corresponding author
Department of Immunology, Tuberculosis Research Centre (ICMR), Mayor V. R. Ramanathan Road, Chetput, Chennai - 600 031, India
Received : 07-11-2001
Accepted : 19-02-2002

and presence or absence of BCG scar were recorded. Biopsy was done wherever applicable. All sera were collected before the start or less than one month of therapy. No samples were collected during treatment.

Out of the 26 cases, 9 were smear and/or culture positive. Three had TB lymphadenititis confirmed by bacteriology or histopathology. Others were clinically diagnosed as having extrapulmonary forms of tuberculosis like, abdominal tuberculosis/Koch's abdomen, TB-cervical adenitis, TB-meningitis, renal tuberculosis, TB synovitis, TB-conjunctivitis etc. The tuberculous etiology was confirmed in them, by their positive response to anti-tuberculous chemotherapy.

Sixty one childhood normal controls (CNHS) included normal school children of less than 15 years of age, Mantoux negative, with or without BCG scar.

Twenty five adult TB patients with positive smear and culture before the starting of chemotherapy, were also included in the study.

Sera

Blood samples were collected, and the serum was separated and stored at -70°C until use.

Culture Filtrate Antigen (CFA)

M. tuberculosis H37Rv culture filtrate antigen (CFA) was prepared as described earlier.[10]

Enzyme linked immunosorbent assay (ELISA)

ELISA was carried out to estimate the IgG, IgA and IgM antibody levels against CFA as described previously.[11] The procedure in brief is as follows:

Polystyrene ELISA plates (Nunc Maxisorp, flat bottom) were coated with l00µl of CFA in the concentration of 5µg/mL in Carbonate buffer, 0.06M, pH 9.6. The plates were incubated with the antigen overnight at 4°C.

The plates were washed four times with phosphate buffered saline (PBS) containing 0.1% Tween 20 (PBST) using automated ELISA Washer (Organon Teknika, Austria). The non-specific sites in the wells were blocked with 1% bovine serum albumin (BSA) for 1h at 37°C. After 4 washes with PBST, the plates were incubated with 1:3200 dilution of the sera from patients and controls. After 1h of incubation at 37°C and washing, antihuman IgG-peroxidase conjugate (Jackson Laboratories, USA), at a dilution of 1:1000 in PBST containing 1% BSA was added as the second antibody. At the end of 1h incubation at 37°C and washing, the colour was developed by the addition of 100µl of the substrate, o-phenylene diamine (OPD) (Sigma Chemical Company, USA) to each well. After arresting the reaction with 50µL of 8N H_2S0_4, the optical density reading was taken in the microplate spectrophotometer (Molecular devices, USA) at 490nm wavelength. The assay was carried out as above for IgA and IgM isotypes also. For this, Antihuman IgA and Antihuman IgM (Jackson Research Laboratories, USA) were used in the dilution of 1:500 and 1:l000 respectively.

The samples to be assayed in a plate were randomly allocated to different wells within the plate and were also coded to conceal the identity of the specimens. Mean absorbance (OD) of CNHS + 2 SD was considered as the cut-off value. Any sample exhibiting absorbance above the cut-off value was classified as positive. Pooled TB sera and PBST buffer were used as the positive and negative controls respectively, in each plate. The cut-off ODs were 0.989,0.486 and 0.644, for IgG, IgA and IgM respectively.

Western blot

M.tubercu1osisH37Rv culture filtrate antigen (CFA) was resolved by 12.5% SDS-PAGE. The proteins from the gel was electro-transferred on to the nitro cellulose paper (NCP) (0.45m pore size, BioRad Laboratories, USA) as per standard procedure.[12] Briefly, after the electrophoretic run, the gels and the NCP were soaked in the blot buffer (0.025M Tris, containing 0.192g glycine and 20% methanol) for equilibration. Electro-transfer was carried out with 0.65A constant current for 1h using the Transblot apparatus (Bio-Rad Laboratories, USA).

The non-reactive sites of the membrane were blocked with 1% BSA (wt/vol) (Sigma Chemical company, USA) in PBS containing 0.3% Tween 20. The NCP was then incubated with 1/100 dilution of patients' sera overnight at 4°C. Further incubation was carried out with 1/4000 dilution of anti-human IgG, A, and M combined Peroxidase conjugate (Jackson Laboratories, USA) for one hour at 37°C. Then the blot was developed with peroxidase substrate 3-3'- Diamino benzidine (Bio-Rad Laboratories, USA) with Nickel chloride (Sigma Chemical Company, USA).

Twenty five sera each from CTB, CNHS and adult TB groups were tested by western blot.

Statistical analysis

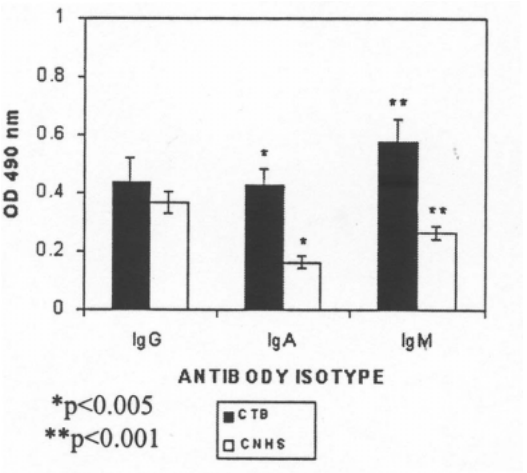
Student's t-test (unpaired) was performed between the mean optical density (OD) of the two categories (CTB and controls) for all the antigens and isotypes to analyse whether significant difference existed among them.

Results

Evaluation of culture filtrate antigen in ELISA

ELISA was carried out for the CTB patients and normal controls using CFA, a mixture of secreted antigens. The mean antibody levels for the three isotypes, were compared. The difference was not significant for IgG, but significant for IgA ($p<0.01$) and IgM ($p<0.001$) between CTB and normals (Fig. 1).

Figure 1 : Antibody levels of CTB and CNHS for CFA antigen



The results obtained with this crude antigen are given in the Table in terms of number and percentage of positivity. Among the CTB patients, two were positive for IgG, nine for IgA and 13 for IgM. Out of 61 controls, two were positive for IgG, four for both IgA and IgM. On combination of results of the three isotypes, a specificity of 93.45% and sensitivity of 57.69% were obtained. The positive and negative predictive values were calculated as 0.79 and 0.84, respectively.

Table : ELISA positivity of CTB and CNHS for CFA antigen

Category	Total	Ig G		Ig G + Ig M		Ig G + IgA + IgM	
	No.	No.	%	No.	%	No.	%
CTB	26	2	7.6	13	50	15	57.69
CNHS	61	2	3.27	4	6.55	4	6.55

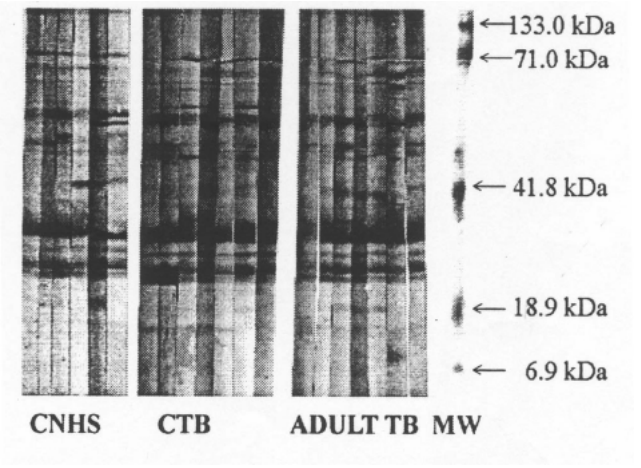
Sensitivity 57.69% Specificity 93.45%

Out of the nine smear and/or culture proven cases, only two were positive for antibodies. Thirteen other cases, which were clinically categorized without bacteriological evidence, were also confirmed by the ELISA.

Western blot analysis

Western blot analysis with CFA was carried out to qualitatively compare the antibody response of CTB, childhood normals and adult TB cases (Fig. 2). Antibodies against 70, 30/31, 27 and 23/24 kDa were found in all the groups, while a number of bands were specifically recognized by the CTB sera. Most of the CTB sera recognized antibodies against 45 kDa (19/25; 75%) 38kDa (23/25; 92%), 19kDa (16/25; 64%) and 16kDa (25/25; 100%). These antibodies were recognized with very less intensity, in smaller number of sera, among the childhood normal controls (45kDa (6/25), 38kDa (5/25), 19kDa (4/25) and 16kDa (8/25).

Figure 2 : Immunoblot with CTB, CNHS and adult TB sera



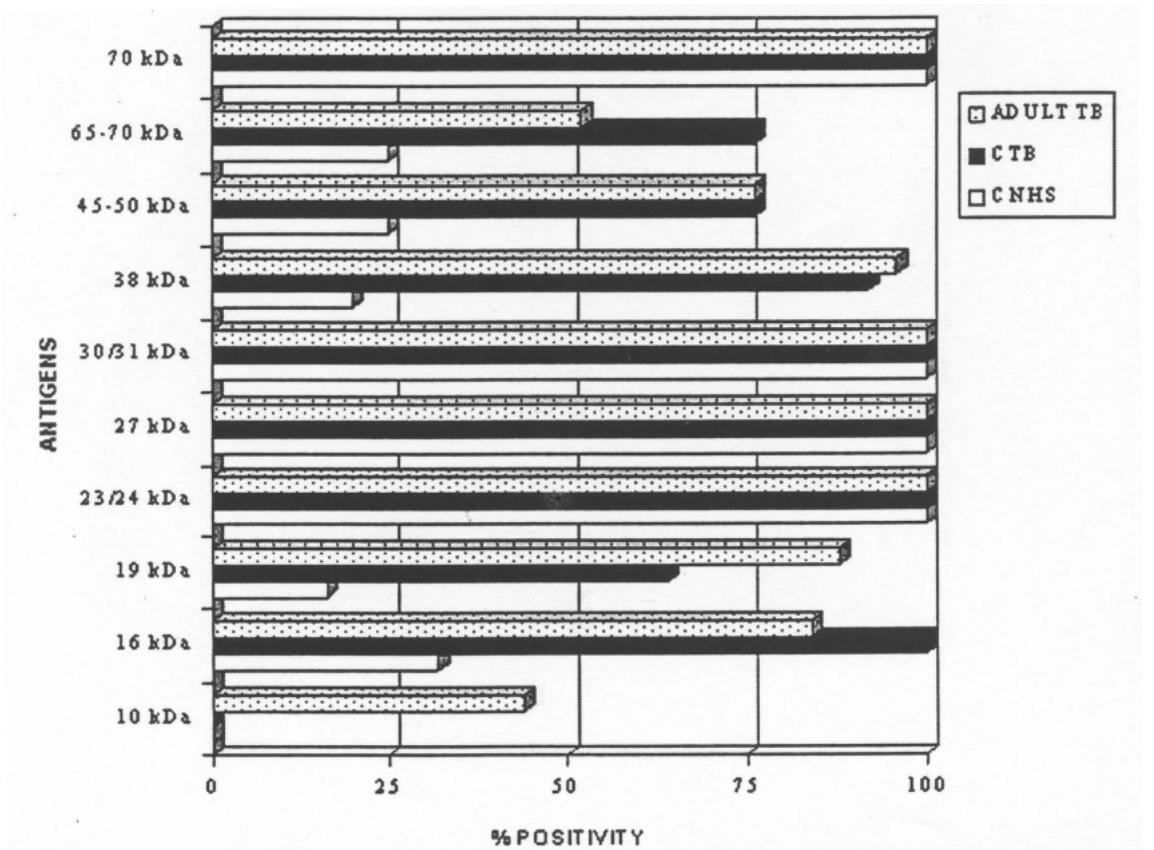
CTB	Childhood tuberculosis patients
CNHS	Childhood normal subjects
Adult TB	Adult tuberculosis patients
MW	Molecular weight marker

When the antibody profile of CTB was compared with that of the adult TB, antibodies in the region of 65kDa and 70kDa region were more intense among the CTB (75%) and less in the adult TB patients (50%). Besides, antibodies against low molecular weight antigens like 10kDa were completely absent in the childhood TB cases and well represented in the adult TB cases (46%). The percentage positivity of the antibody recognition has been represented in the graphical form in the Fig. 3.

Discussion

Many investigators have previously used crude,[3,4] semi-purified[5-7] and purified mycobacterial antigens[7-9] for serodiagnosis of childhood tuberculosis. Using mycobacterial sonicates, Rosen showed poor sensitivity (21%) and specificity (40%), due to cross-reactivity with BCG[3]. Barrera *et al*, using PPD got a much higher sensitivity of 51% with a specificity of 98% in bacteriologically confirmed cases of childhood tuberculosis patient.[4]

Figure 3 : Antibody profile of CTB, CNHS and adult TB in immunoblot



Using the CFA of *M. tuberculosis* H37Rv, we obtained a sensitivity of 57.7% with a specificity of 93.5%, when a combination of all three isotypes (IgG+IgA+IgM) was considered. These results are comparatively higher than in any other earlier studies conducted with crude antigens.[3-5]

Positivity for IgG antibodies alone is very less, while number of sera positive for IgM is higher. Since childhood tuberculosis is a form of primary progressive disease, the presence of greater IgM than IgG, as a primary response is expected. Similar results have been obtained in an earlier study[7], using the same sera, with A60 antigen. Additionally, the IgG antibodies may also have been trapped in circulating immune complexes (CIC) and not present in the free form. Indeed, we could measure CIC bound antibodies in adult tuberculosis sera, even when they were negative for free antibodies, against purified 38kDa antigen[11] as well as 30 and 16kDa antigens.[13]

The reason for elevated IgA response is not clear. IgA has traditionally been regarded as a non-inflammatory antibody. While this may be true of secretory IgA, new insight into the role of serum IgA implicates it in pro-inflammatory reactions.[14] Our previous studies on bronchoalveolar lavage,[15] as well as sera,[11] have demonstrated elevated IgA response, locally and systemically. Also, the synthesis of only IgG or IgA antibodies among patients with tuberculosis and the need for joint analysis of both for better diagnostic performance, has been repeatedly emphasized by those who have reported on antigen A60.[5,6]

An alternate approach was made towards analysis of antibody profiles among the childhood tuberculosis and the normal controls using immunoblot analysis against the CFA. Adult pulmonary tuberculosis patients were also included to compare the differences in childhood and adult response, if any. From our experiments, we observed that children generally do not produce antibodies of lower molecular weight like l0kDa and below. Antibodies around 16, 19, 38 and 45kDa region may be useful in differentiating the CTB patients from childhood normal controls by immunoblot assay. Antibodies in the region of 65kDa were predominantly seen among the CTB and not in the adult patients.

The reasons of absence of recognition of low molecular weight antigens by CTB sera are not clear. Bothamley[16] has reported that anti-65kDa antibodies are elevated in CTB. Our blot results are in agreement with this observation. It has been observed that while in post-primary tuberculosis like adult tuberculosis, the antibody response is mainly directed against secreted antigens, in

primary tuberculosis (CTB), the prominent antibody response is against cytosolic antigens.[16] It may be one of the reasons for absence of recognition of low molecular weight secreted CFA proteins.

Therefore, it is obvious from this study that combination of results of three isotype (IgG, A & M), was found to increase the sensitivity of the assay without significantly compromising the specificity, even using crude antigen. Even though only two out of the nine smear and/or culture proven cases were positive for antibodies, thirteen other cases, which were clinically categorized without bacteriological evidence, were also confirmed by the ELISA.

Antibodies against 16, 19, 38 and 45kDa region may be useful in diagnosis of childhood tuberculosis as seen in immunoblot.

Acknowledgement

We thank Dr. Sowmya Swaminathan, Tuberculosis Research Centre and Dr. Vijayasekaran, Institute for Child Health, Chennai, for providing the samples. Financial support provided by the Indo-US Vaccine Action Programme, through the Department of Biotechnology, Government of India, is gratefully acknowledged.

References

1. Kochi A. The global tuberculosis situation and the new control strategy of the World Health Organization. *Tubercle* 1991;**72**:1-6.
2. Singh V. Available tests for childhood tuberculosis. *IndianPediatrics* 1997;**34**:965-966.
3. Rosen E. The diagnostic value of an enzyme-linked immunosorbent assay using absorbed mycobacterial sonicates in children. *Tubercle* 1990;**71**:127-130.
4. Barrera L, Kiceli I, Ritaco V, Torrea B, Broglia B, Botta R. Detection of circulating antibodies to purified protein derivative by enzyme linked immunosorbent assay: its potential for the rapid diagnosis of tuberculosis. *Pediatr Infect Dis J* 1989;**8**:763-767.
5. Delcourt, C, Gobin J, Gaillard J, de Blic J, Vernon M, Schinmann P. Value of ELISA using Antigen 60 for the diagnosis of tuberculosis in children. *Chest* 1993;**104**:393-398.
6. Gupta S, Bhatia R, Datta KK. Serological diagnosis of childhood tuberculosis by estimation of mycobacterial antigen 60-specific immunoglobulins in the serum. *TubercLungDis* 1997;**78**:21-27.
7. Swaminathan S, Umadevi P, Shantha S, Radhakrishnan A, Datta M. Sero Diagnosis of Tuberculosis in children using two Elisa kits. *Indian JPediatrics* 1999;**66**:837-842.
8. Alde S, Pinasco HM, Pelosi FR, Budani HF, Palma-Beltran OH, Gonzalez-Montaner LJ. Evaluation of an Enzyme-linked Immunosorbent Assay (ELISA) using an IgG antibody to *Mycobacterium tuberculosis* Antigen 5 in the diagnosis of active tuberculosis in children. *Am Rev Respir Dis* 1989;**139**:748-751.
9. Zheng Y, Wang RH, Lin YZ, Daniel T. Clinical evaluation of the diagnostic value of measuring IgG antibody to 3 mycobacterial antigen preparations in the capillary blood of children with tuberculosis and control subjects. *TubercLungDis* 1994;**75**:366-370.
10. Senthil Kumar KS, Raja A, Uma Devi KR, Paranjape RS. Production and characterization of monoclonal antibodies to *Mycobacterium tuberculosis*. *Indian JMedRes* 2000;**112**:37-46.
11. Uma Devi KR, Ramalingam B, Brennan PJ, Narayanan PR, Raja A. Specific and early detection of IgG, A and M antibodies to *M.tuberculosis* 38kDa antigen in pulmonary tuberculosis. *Tuberculosis* 2001;**81**:249-253.
12. Towbin H, Stahelin T, Gorden J. Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose sheets: procedure and application. *Pro NatlAcadSci* USA. 1979;**76**:4350-4354.
13. Alamelu Raja, Uma Devi KR., Ramalingam B, Brennan PJ. Immunoglobulin G, A and M response in serum and circulating immune complexes against 16kDa antigen of *M.tuberculosis*. Clinical and *Diagnostic Laboratory Immunology* 2002; March Issue (In press).
14. Egmond MV, Damen CA, van Spriel AB, Vidarsson G, van Garderen E, van de Winkel JGJ. IgA and IgA Fc receptor. *TrendsImmunol* 2001;**22**:205-211.
15. Raja A, Baughman R P, Daniel T M. The detection by immunoassay of antibody to mycobacterial antigens and mycobacterial antigens in bronchoalveolar lavage fluid from patients with tuberculosis and control subjects. *Chest* 1988;**94**:133-137.
16. Bothamley G. Serological diagnosis of tuberculosis. *EurRespirJ* 1995;**8** suppl.:676s-688s.