

# A comparative evaluation of ELISA test employing antigen 85C with ZN staining, culture and PCR in the diagnosis of Tuberculosis in children

Kumar N<sup>1</sup>, Dayal R<sup>2</sup>, Joshi B<sup>3</sup>, Chauhan DS<sup>4</sup>, Singh HB<sup>5</sup>, Jaiswal A<sup>6</sup>, Shankar H<sup>7</sup>

<sup>1</sup>Dr. Neeraj Kumar, Associate Professor, department of Pediatrics, S. N. Medical College Agra, India. <sup>2</sup>Dr. R. Dayal, Professor & head department of Pediatrics, S.N. Medical College Agra, India. <sup>3</sup>Mr. B. Joshi, Scientist E /Deputy Director, National JALMA Institute for Leprosy and Other Microbial diseases Agra, India. <sup>4</sup>Dr. D. S. Chauhan, Head of Micro & Molecular Biology, National JALMA Institute for Leprosy and Other Microbial diseases Agra, India. <sup>5</sup>H. B. Singh, Scientist B, National JALMA Institute for Leprosy and Other Microbial diseases Agra, India. <sup>6</sup>Mr. Dr. A. jaiswal, Junior Resident department of Pediatrics, S. N. Medical College Agra, India. <sup>7</sup>Mr. H. Shankar, Research scholar, National JALMA Institute for Leprosy and Other Microbial diseases Agra, UP, India.

**Address for correspondence:** Dr. Neeraj Yadav, Email: [neerajyadu@rediffmail.com](mailto:neerajyadu@rediffmail.com)

## Abstract

**Objectives:** Childhood tuberculosis is difficult to diagnose. A rapid, simple and relatively inexpensive diagnostic test will be crucial to future control efforts. Therefore, diagnostic potential of secretory antigen 85C in human sera specimen, CSF and other body fluids of *Mycobacterium tuberculosis* in childhood tuberculosis cases were evaluated using ELISA technique and reactivity was compared with the gold standards (Ziehl Neelsen staining, BACTEC culture) and IS6110 targeted PCR. **Methods:** In the present study, 73 fresh, untreated childhood tuberculosis (TB) cases under 18 years of age out of these 27 had pulmonary tuberculosis and 46 patient had extra pulmonary tuberculosis. Twenty healthy children were included. **Results:** ELISA with Antigen 85C in sera was positive in 88.89% and 80.43% of pulmonary and extrapulmonary cases, respectively. Overall sensitivity and specificity were 83.56% and 65%, ( $p < 0.001$ ). ELISA in different body fluids were positive in 0% and 36.96% of the pulmonary and extrapulmonary cases, respectively. Overall sensitivity and specificity were 23.29% and 90% respectively ( $p > 0.05$ ). PCR targeting IS6110 was positive in 96.30% and 65.22% of pulmonary and extrapulmonary cases, respectively. Overall sensitivity and specificity were 76.71% and 90%, respectively. **Conclusions:** Our findings demonstrates the potential of Ag 85C in sera in the detection of antibody in childhood TB cases and this antigen showed good concordance with PCR positivity.

**Key words:** Tuberculosis, ZN staining, antigen 85C, PCR.

## Introduction

Tuberculosis (TB) is a global public health hazard. Out of the total 14 million prevalent cases, most of cases were found in the South-East Asia, African and Western Pacific regions (35%, 30% and 20%, respectively). An estimated 11–13% of incident cases are HIV-positive [1]. Recently, estimated cases among children have been on the rise [2]. Although paediatric age group cases represent a small proportion of all tuberculosis cases yet the infected children act as a reservoir from which many adult patients may arise [3]. Identification of the micro-organism in the body

secretions or body tissues from the patient is very important for the diagnosis of the tuberculosis; however, this is not always feasible in children because they rarely produce sputum and hence microscopic demonstration of the bacilli in the sputum yields mostly negative results [4]. Tuberculosis in children appear as extra pulmonary, hence it becomes very difficult to confirm bacteriological diagnosis. Diagnosis in children relies on tuberculin skin test (Monteux test), chest radiograph and clinical signs and symptoms. However, clinical features may be non-specific, tuberculin skin test and chest radiograph can be difficult to interpret. Other techniques such as BACTEC, fluorescent antibody test, gas chromatography, DNA hybridization, RIA and PCR are sensitive but require well-established laboratory and costly equipments [5]. Therefore, a

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sensitive, less expensive and simple sero-diagnostic test will help in early diagnosis leading to a reduction in mortality among children with tuberculosis and further transmission of this disease.

Enzyme-linked immunosorbent assay (ELISA) is a potentially valuable technique and simple to perform. Crude as well purified forms of antigens of *M. tuberculosis* have been employed in the ELISA in an attempt to increase both the sensitivity and specificity in both children and adults [6-8]. The heterogeneous immune response of patients to a variety of antigens with limited specificity to a single antigen, indicating the variations among individuals as well as disease stage [9]. So far, there is no single antigen, which can be used to diagnose tuberculosis. Improvement in test performance has been reported by using a mixture of multiple antigens in the assay [10]. Culture filtrate proteins (CFPs) of *M. tuberculosis* are among the earliest antigens encountered by the host immune system and have been shown to be immunodominant. CFPs such as CFP-10, ESAT-6 and Ag85 complex are being extensively evaluated for their role as inducer of T cell responses in active adult TB cases [11]. The antibody profile status to secreted antigens has not been investigated adequately in children. A recent study reported utility of ESAT-6 in children [12]. Raja *et al.* have found 30 kDa antigen to be highly sensitive and specific in the serological assay when IgG, IgM and IgA antibodies were measured for the diagnosis of tuberculosis in paediatric cases [13].

In this study, we have evaluated the reactivity of Antigen 85C in pulmonary and extrapulmonary tuberculosis cases and healthy children in comparison to ZN staining, BACTEC culture, and PCR IS6110 test. Furthermore, the study was also extended to correlate diagnosis potential of Antigen 85C in both sera as well as various body fluids in diagnosis of tuberculosis.

## Materials and Methods

**Study subjects.** Seventy three children of either sex less than 18 years of age, who were clinically diagnosed as having tuberculosis were chosen from the outpatient department and ward of Department of Paediatrics, S.N. Medical College, Agra. Informed consent was obtained from patients and their guardians. Out of these patients, 27 children had pulmonary TB (PTB) and 46 had extra pulmonary TB (EPTB). Cases were classified as per criterion laid down in the consensus statement of Indian Association of Paediatrics working group [14]. All these cases were subjected to detailed clinical history,

thorough physical examination and routine and specific laboratory investigations. Sputum samples/ gastric aspirate/ pleural fluid (which were feasible to obtain) from PTB cases, lymph node aspirate from tuberculous lymphnode, CSF from tuberculous meningitis and ascitic fluid from abdominal tuberculosis were subjected to Ziehl Nielsen staining for acid fast bacillus (AFB), culture for TB at the National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Agra for establishing a provisional diagnosis for all the cases. Presence or absence of BCG scar, tuberculin skin test to purified protein derivative (PPD) and history of household contact were recorded for all the patients. Venous blood from these cases was collected before the start or before completion of 1 month of therapy. Twenty healthy children with non-tuberculous involvement of lung, lymph nodes, abdomen and central nervous system, with or without BCG scar were also included as healthy and disease controls, respectively. Healthy controls comprised healthy childhood contacts of TB patients who were tuberculin skin test negative and were absolutely normal on clinical examination which included an assessment of growth and development as well. Serum and different body fluids were separated out and stored at -20 °C till used. This study was undertaken after obtaining clearance from Institutional ethical committee following the guidelines of Indian Council of Medical Research.

**Antigens-** Recombinant antigens: Antigen 85C was procured from the laboratory of Dr. John T. Belisle, Colorado State University, USA. Purity of this antigen was confirmed by SDS-PAGE and silver staining (Quality Control Record enclosed along with antigen details). The identification of secreted proteins of *M. Tuberculosis* open the way to studies on their sub cellular localization in *M. Tuberculosis* and to the immunological characterization of these proteins to define their for immunological diagnosis of tuberculosis [15].

**IgG antibody detection by ELISA-** It was carried out to estimate the IgG antibody levels against recombinant Ag 85C. Briefly, polystyrene microtitre plates (flat bottom, Nunc Maxisorp, Roskilde, Denmark) was coated with 100 µl of Ag 85C (25 ng/ml), in carbonate bi-carbonate buffer 0.05 M, pH 9.6. The concentration mentioned was optimized by chequerboard titration. The plates were incubated overnight at 37 °C in a humid chamber. After incubation, the plates were washed with PBS (phosphate-buffered saline)

containing 0.05% Tween 20 (PBST) three times (3x) and blocked with 300  $\mu$ l/well of blocking buffer (1% BSA in PBS) for 1 h at 37 °C. Plates were washed (3x) after incubation and 100  $\mu$ l of samples (1:100 diluted serum and undiluted different body fluids) were added in duplicate onto the wells and plates were incubated for 2 h at 37 °C.

After washing anti-human IgG peroxidase conjugated (Sigma, St Louis, MO, USA) were added to each well at the dilution of 1:10,000 in 1% BSA in PBST. After incubation for 1 h at 37 °C and washing (3x), plates were developed by adding 100  $\mu$ l/well of chromogen ortho-phenylene diamine (OPD) (1 tablet of 5mg and 50  $\mu$ l H<sub>2</sub>O<sub>2</sub> in 10 ml distilled water). The reaction was stopped by adding 50  $\mu$ l/well of stop solution (7% H<sub>2</sub>SO<sub>4</sub>). Absorbance was read at 492 nm in Spectramax

## Results

Antibody reactivity of children with active TB cases in children were evaluated by ELISA using Ag 85C. Antibody reactivity of healthy children was also recorded. Cutoff was selected for antigen 85C at the point which showed best accuracy, sensitivity and specificity. Antibody response in children suffering from diseases and healthy children were compared to ZN staining, BACTEC culture, and PCR IS6110 test. Ziehl-neelsen staining of the respective sample demonstrated Acid Fast Bacilli in 5 out of 27 (18.52) and 0% in pulmonary and extra pulmonary cases respectively. no statistically significant difference was The BacT/Alert 3D system culture for m. Tuberculosis was positive in 11 out of 27 (40.74%) and 20 out of 46 (43.48%) of pulmonary and extrapulmonary cases respectively.

**Table-1: Comparative evaluation of sensitivity and specificity of various diagnostic methods in pulmonary and extrapulmonary systems.**

System Studied		ZN staining positive cases (%)	BacT/Alert culture positive cases (%)	PCR (IS6110) positive cases (%)	ELISA (Ag 85C) in sera positive cases (%)	ELISA (Ag85C) in different body fluids positive cases (%)
Pulmonary (n=27)	Sensitivity	18.52	40.74	96.30	88.89	0
	Specificity	100	100	83.33	83.33	100
Extra-pulmonary (n=46)	Sensitivity	0	43.48	65.22	80.43	39.96
	Specificity	100	100	92.86	57.14	85.71

**Antibody response to Ag85 C in sera:** Antibody reactivity to Ag 85C in sera was determined. ELISA with Ag 85C in sera was positive in 88.89% and 80.43% of the pulmonary and extrapulmonary cases, respectively. A total of 83.56% cases in the study group tested positive. In healthy control group, 16.67% and 42.86% of pulmonary and extra pulmonary cases tested positive. Overall sensitivity and specificity were 83.56% and 65%, respectively (p<0.001).

**Antibody response to Ag 85C in Different Body Fluids:** ELISA with Ag 85C in different body fluids was positive in 0% and 36.96% of the pulmonary and extrapulmonary cases, respectively. A total of 23.29% cases in the study group tested positive. In the healthy control group, 0% and 14.29% of pulmonary and extrapulmonary cases tested positive. Overall sensitivity and specificity were 23.29% and 90% respectively (p>0.05).

**Polymerase chain reaction:** PCR targeting IS6110 was positive in 96.30% and 65.22% of pulmonary and extra pulmonary cases, respectively. A total of 76.71% cases in the study group tested positive. In the control group all the

M2 plate reader (Molecular Devices, Sunnyvale, CA, USA).

**Polymerase chain reaction-** DNA was isolated by a physicochemical method using lysozyme and proteinase K being routinely used in the Microbiology and Molecular Biology laboratory of this Institute [16]. DNA amplification was performed using IS1 and IS2 that specifically amplified 123 bp fragment of IS 6110 [17].

**Statistical analysis-** The relation between the sensitivity and specificity at various cutoff of antigen 85C was shown in comparison to ZN staining, BACTEC culture and PCR (IS6110). Positivity of Ag85C in sera and different body fluids from tuberculosis cases and controls were also correlated.

patients were negative. Overall sensitivity and specificity were 76.71% and 90%, respectively ( $p < 0.001$ ). When PCR results were compared with ELISA using Ag 85C, a very good concordance was observed as 81.27% of the patients positive by PCR were having antibody to Ag85C in sera.

## Discussion

Ever since the discovery Koch's bacillus, the diagnosis of tuberculosis still largely depend on clinical examination, radiographic finding and laboratory test. Secretory antigens of *M. tuberculosis* have been reported to be immunogenic and hence are thought to have diagnostic potential [18]. Humoral response to Ag 85C in tuberculosis has been reported [19-21]. Hence utility of this antigen in the diagnosis of TB has been envisaged. In the present study, antibody reactivity to secretory antigen 85C was analysed in serum and different body fluids in various types of paediatric TB patients and compared with the antibody response of healthy children. We observed highest sensitivity and specificity of 83.56% and 65%, respectively, using Ag 85C in sera. however, sensitivity and specificity of 23.29% and 90% was observed using Ag 85C in different body fluids. Similar to our finding in children G Kumar *et al.* have also noted sensitivity of 89.77% using Ag 85C in paediatrics TB cases; however, they have noted specificity of 92% [19]. Ag 85C has been reported to be secreted early in the infection in adult cases [22]. Role of this antigen in the serodiagnosis of the patients before they develop cavitation has been reported by these authors. Our study confirms the finding of serodiagnostic potential of Ag85C of the above reports in the paediatric age group also. Ag85 homologues are present in non-pathogenic mycobacteria and in corynebacteria [23]. We assume that the low specificity noted in our study could be due to the presence of cross-reactive antibodies to these non-pathogenic strains of bacteria. Differences noted in this study could be due to the paediatric population having different disease pathology. More numbers of smear negative patients were found to be reactive to these antigens than the earlier report [24]. Raja *et al.* have reported sensitivity of 65.4% using 30 kDa antigen; however, sensitivity was increased by combining IgG, IgM and IgA response in childhood tuberculosis [25]. Antibody response to antigen 85C was compared with the PCR positivity. We observed very good concordance with the result of PCR and antibody response to Ag 85C in sera. The present study explores the antibody profiles of paediatric TB patients to secretory antigen 85 C in sera and different body fluids. Ag 85C in sera was found to be most promising. A good concordance was also noted with PCR positive patients using this antigen. It could be useful to reduce

the heterogeneous antibody response of childhood tuberculosis cases for TB diagnosis in different regions of an endemic country such as India.

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