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Diagnostic Potential of Ag85C in Comparison to Various Secretory Antigens for Childhood Tuberculosis

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Abstract

Childhood tuberculosis is difficult to diagnose. A rapid, simple and relatively inexpensive diagnostic test will be crucial to future control efforts. Therefore, efficacy and diagnostic potential of different secretory antigens of *Mycobacterium tuberculosis* (CFP-10, Ag85complex, Ag85 A, B, C) and their combinations along with ESAT-6 in the detection of antibody profiles of childhood tuberculosis cases were evaluated using ELISA technique and reactivity was compared with the gold standards (smear, culture and IS6110 targeted PCR). In the present study, 88 fresh, untreated childhood tuberculosis (TB) cases, 17 children undergoing anti-tubercular therapy, 17 children having disease other than TB and 25 healthy children were included. ROC curve analysis was used to calculate the sensitivity and specificity of each antigen for antibody detection. Ag85C was found to be showing highest sensitivity of 89.77% and specificity of 92% among all the antigens used ($P < 0.0001$). Positivity with antigen was 95% in smear and culture negative patients. Antibody reactivity was noted in 92.62% of patients who were positive for IS6110 by PCR. Cocktail of all the antigens showed 67.1% sensitivity and 80% of specificity. Sensitivity of 29.55%, 57.95%, 64.77% and specificity of 80%, 72%, 64% was observed using CFP-10, Ag85complex and Ag85B. Low reactivity of 31.82% in patients and least specificity of 24% was noted with Ag85A. Our finding demonstrates the potential of Ag85C in the detection of antibody in childhood TB cases and this antigen showed good concordance with PCR positivity.

Introduction

Tuberculosis (TB) is a global public health hazard. One-third of global population is estimated to be infected with *Mycobacterium tuberculosis* [1]. Recently, estimated cases among children have been on the rise [2]. Although paediatric cases represent a small proportion of all tuberculosis cases yet the infected children act as a reservoir from which many adult cases may arise [3]. Identification of the micro-organism in the secretion or tissues from the patient is the mainstay for the diagnosis of the tuberculosis; however, this is not always feasible in children because paediatric patients rarely produce sputum and hence microscopic demonstration of the bacilli in the sputum yields mostly negative results [4]. Moreover, in most of the children the tuberculosis cases appear as extra pulmonary, hence it becomes more difficult to confirm bacteriologically. Diagnosis in children relies on tuberculin skin testing, chest radiograph and clinical signs and symptoms. However, clinical symptoms may be non-specific, skin testing and chest radiograph can be difficult to interpret. Other techniques such as BACTEC, fluorescent antibody test, gas chromatography, DNA hybridization, PCR and RIA are sensitive but require well-established laboratory and costly equipments [5]. Therefore, a sensitive, cost-effective 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 improve both the sensitivity and specificity in children as well as in adults [6–8]. These reports reveal 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 antigen 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 ESAT-6, CFP-10 and Ag85complex are being extensively evaluated for their role as inducer of T cell responses in active adult TB cases [11]. However, antibody profile status to secreted antigens has not been investigated adequately in children. We have recently reported utility of ESAT-6 in paediatric patients [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 children [13].

In this study, we have evaluated the antibody reactivity in various groups of children (active TB, cases who have taken minimum of 2 months of anti-tubercular treatment (ATT), children suffering from other diseases and healthy children) to recombinant CFP-10, Ag85complex, individual components of Ag85 (A, B, C) and a cocktail of these antigens.

Materials and methods

Study subjects. Eighty-eight children of either sex under 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, 36 children had pulmonary TB (PTB) and 52 had extra pulmonary TB (EPTB). EPTB patients included tubercular lymphadenitis (TBL) (20), abdominal TB (11), TB meningitis (TBM) (20) and miliary TB (1). Cases were classified according to the criterion laid down in the consensus statement of Indian Association of Paediatrics working group [14]. All the cases were subjected to detailed clinical history, thorough physical examination and routine and specific laboratory investigations. Gastric aspirate/sputum samples/pleural fluid (which were feasible to obtain) from PTB cases, lymph node aspirate from TBL, CSF from TBM and ascitic fluid from abdominal TB 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, 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. Blood samples were also collected from 17 children with TB who had received anti-tubercular treatment (ATT) for minimum of 2 months. Twenty five healthy children and 17 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 Montoux negative and were absolutely normal on clinical examination which included an assessment of growth and development as well. Serum was separated out and stored at −20 °C till used. Study was undertaken after obtaining clearance from Institutional ethical committee following the guidelines of Indian Council of Medical Research.

Antigens. Recombinant antigens: ESAT-6, CFP-10, Ag85complex and Ag85 A, B, C were procured from the laboratory of Dr. John T. Belisle, Colorado State University, USA (under the NIH Contract HHSN266200400091C/ADB Contract NOI-AI-40091). Purity of these antigens was confirmed by SDS-PAGE and silver staining (Quality Control Record enclosed along with antigen details).

IgG antibody detection by ELISA. It was carried out to estimate the IgG antibody levels against recombinant CFP-10, Ag85 complex, Ag85 A, B, C and cocktail of all these antigens which also included ESAT-6. Briefly, polystyrene microtitre plates (flat bottom, Nunc Maxisorp, Roskilde, Denmark) were coated with 100 μl of CFP-10 (25 ng/ml), Ag85complex (25 ng/ml), Ag85A (25 ng/ml), Ag85C (25 ng/ml), Ag85B (12.5 ng/ml) and cocktail of these antigens at the same concentration along with ESAT-6 (50 ng/ml), in carbonate bi-carbonate buffer 0.05 M, pH 9.6. All the concentrations mentioned were 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 200 μl/well of blocking buffer (1% BSA in PBS) for 1 h at 37 °C. Plates were washed (3x) after incubation and 100 μl of diluted sera in 1% BSA in PBST (1:200 for CFP-10, Ag85complex, Ag85B and cocktail, 1:100 for Ag85C and 1:50 for Ag85A) 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 μl/well of chromogen ortho-phenylene diamine (OPD) (Sigma) at a concentration 0.5 mg/ml of DW and 0.2 μl/ml H_2O_2 (Qualigens, Mumbai, India). The reaction was stopped by adding 50 μl/well of stop solution (7% H_2SO_4). Absorbance was read at 492 nm in Spectramax 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 our Institute [15]. DNA amplification was performed using IS1 (5′CCTGCGAG-CGTAGGCGTCGG3′) and IS2 (5′CTCGTCCAGCG-CCGCTTCGG3′) that specifically amplified 123 bp fragment of IS 6110 [16]. Briefly, 5 μl of DNA (extracted from sputum and other specimens as described in Materials and methods) was amplified using 1.5 Unit of Taq polymerase in a 50 μl reaction mixture containing 200 μM dNTP mix and 200 ng each forward and reverse primer. DNA amplification was performed for 35 cycles in a thermal cycler (GeneAmp PCR system 9700; Applied Biosystems, Foster City, CA, USA) by using following programme: initial denaturation for 5 min at 94 °C, annealing at 70 °C and extension at 72 °C for 1.5 min with a final extension of 10 min at 72 °C. Amplicons were checked for the amplification of *M. tuberculosis* specific IS6110 sequence by electrophoresis on 1.2% agarose gel.

Statistical analysis. Software STATA-7 (Strata Corporation, College Station, TX, USA) was used for statistical analysis. ROC (receiver operator characteristics) curve was constructed to describe the relation between the sensitivity and specificity at various cutoff of antigens specific IgG. Scattergram was plotted using GRAPHPAD software prism version 3.02 (GraphPad Software, Inc., San Diego, CA, USA).

Results

Antibody reactivity of children with active TB cases, children undergoing ATT was evaluated by ELISA using CFP-10, Ag85complex, Ag85 A, B, C and cocktail of all these antigens and ESAT-6. Antibody reactivity of healthy children and children with other diseases was also recorded. ROC curve analysis of antibody levels was done for determining the sensitivity and specificity of each antigen. Cutoff was selected for each antigen at the point which showed best accuracy (correctly classified individuals to their groups), sensitivity and specificity. Antibody response of active TB cases, children during ATT, children having diseases other than TB and healthy children was compared.

Antibody response to CFP-10

Sensitivity 29.55% and specificity of 80% was noted in case of CFP-10 using a cutoff of 0.075 (Table 1). At this cutoff, 40.71% of accuracy was noted. Antibody response was noted in 32.35% of patients who were negative by culture and smear examination (Table 2). Only 15% patients with TBM, 30% of TBL, 33.33% of PTB and 36.36% of abdominal TB cases and one miliary TB patient were showing reactivity to CFP-10. No children undergoing treatment was showing antibody reactivity but 23.52% of children with other diseases were showing reactivity above the cutoff using this antigen (Fig. 1).

Antibody response to Ag85 complex and its components

Antibody reactivity to Ag85complex and its individual components A, B, C was also determined. The sensitivity and specificity of the assay was 57.95% and 72%, respectively, using a cutoff value of 0.068 for Ag85complex (Table 1). Accuracy of the assay was 61.1%, 58.82% of patients both negative by smear and culture examinations were showing antibody reactivity. 63.63%, 58.33%, 55% and 60% of abdominal PTB, TBL and TBM patients, respectively, were showing antibody reactivity. One patient with miliary TB did not show any reactivity. 35.29% of treated children and 52.94% of children with other disease were positive for Ag85complex antibody (Fig. 1). Low reactivity (31.82%) to Ag85A and specificity of 24% was noted at a cutoff of 0.070 and it was found to be only 30.1% accurate (Table 1). Only 36.76% of children negative by smear and culture examination were positive by ELISA (Table 2). Antibody response above cutoff was seen in 18.18% of abdominal TB, 27.77% of PTB, 45% of TBL and 45% of TBM patients.

Table 1 Sensitivity and specificity of various secretory antigens by ROC curve analysis.

Antigen	Cut off	Sensitivity	Specificity	+PV	−PV	Accuracy (%)	ROC	*P* value area (0.5)
CFP-10	0.075	29.55	80.0	83.9	24.3	40.71	0.558	0.3653
Ag85 complex	0.068	57.95	72.0	87.9	32.7	61.1	0.645	0.014
Ag85A	0.070	31.82	24.0	39.6	93.3	30.1	0.228	<0.0001
Ag85B	0.050	64.77	64.0	86.4	34.0	64.60	0.611	0.070
Ag85C	0.043	89.77	92.0	97.5	71.9	90.27	0.975	<0.0001*#
Cocktail	0.070	67.1	80.0	92.2	40.8	69.91	0.796	<0.0001+

*P < 0.0001 when area under ROC of Ag85C was compared with Ag85 complex.
#P = 0.0002 when area under ROC of Ag85C was compared with cocktail.
+P = 0.055 when area under ROC of Ag85complex was compared with cocktail.

Table 2 Correlation of antibody response of various antigen with the result of culture and smear examination.

	Smear+ culture+ (n = 3)		Smear− culture+ (n = 10)		Smear+ culture− (n = 7)		Smear− culture−(n = 68)	
Antigen	Total + ve	% +ve	Total +ve	% +ve	Total +ve	% +ve	Total +ve	% +ve
CFP-10	1	33.33	3	30	0	0	22	32.35
Ag85complex	1	33.33	6	60	4	57.14	40	58.82
Ag85A	2	66.67	1	10	2	28.57	25	36.76
Ag85B	0	0	6	60	5	71.43	46	67.65
Ag85C	3	100	9	90	6	85.71	57	95.00
Cocktail	3	100	8	80	4	57.14	44	64.71

+ve = above cutoff.

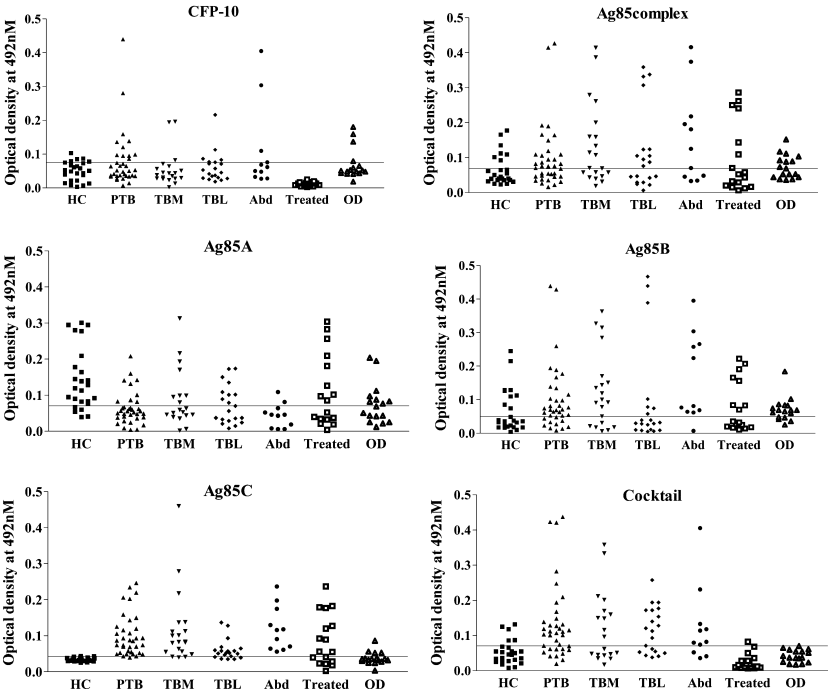

Figure 1 Scatter diagram of antibody response of various groups of children to secretory antigens. Dotted bar shows optimum cutoff point obtained by ROC curve analysis. Healthy control (HC), pulmonary tuberculosis (PTB), tuberculosis meningitis (TBM), tuberculosis lymphadenitis (TBL), abdominal (Abd), treated, other diseases control (OD).

52.94% of children undergoing treatment and 47.1% of children with other diseases were showing reactivity to this antigen (Fig. 1). On the other hand, sensitivity of 64.77% and specificity of 64% were found using cutoff of 0.050 when Ag85B was used (Table 1) and this assay was 64.6% accurate. This antigen could detect 67.65% patients by ELISA who were neither positive by smear nor by culture examination (Table 2). 90.90% of abdominal TB, 72.22% of PTB, 70% of TBM, 35% of TBL patients were showing antibody response to Ag85B. 47.1% of children undergoing treatment and 70.58% of children with other disease were showing antibody positivity (Fig. 1). Ag85C was 89.77% sensitive and 92% specific at the cutoff 0.043 and accuracy of the test was found to be 90.27% (Table 1). 95% of patients who were neither positive by smear nor by culture examination were showing antibody reactivity to this antigen (Table 2). Positive response was observed in all the abdominal and in one miliary TB patient. On the other hand, 91.66% of PTB, 70% of TBL, 85% of TBM patients and 58.82% of children undergoing treatment showed reactivity to Ag85C. Only 23.52% of children with other diseases were showing antibody response above cutoff (Fig. 1).

Antibody response to antigen cocktail

All these sera were subjected to ELISA using the cocktail (Cocktail 1) of all these antigens described above and ESAT-6. Sensitivity with this mixture was 67.1%, however, the specificity was found to be 80.0% using cutoff of 0.07 (Table 1). 69.91% of accuracy of the test was noted. 64.71% patients who were neither positive by smear nor culture examination were showing reactivity to cocktail (Table 2). Positivity of 72.72% in abdominal TB, 65% in TBL, 69.44% in PTB and 55% in TBM was noted. One miliary TB patient was showing reactiv-

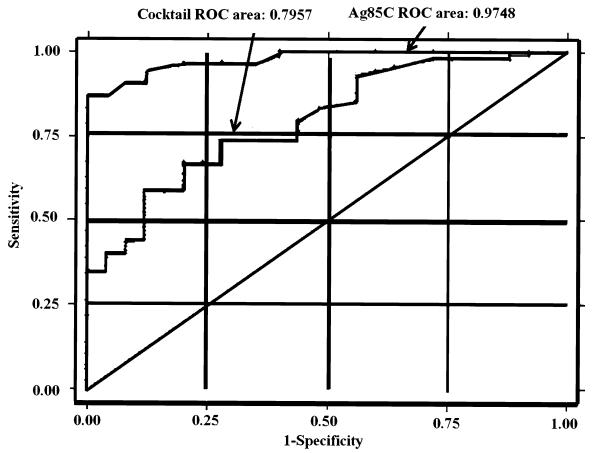

Figure 2 ROC curve of Ag85C and cocktail of antigens showing sensitivity and specificity.

ity above cutoff. Reactivity of only 5.8% was found in children undergoing treatment and none of the disease control showed antibody response above cutoff (Fig. 1). Since Ag85A was not found to be showing good reactivity, a cocktail was made (cocktail 2) without this antigen and reactivity was recorded. No difference was found in the antibody response between cocktail 1 and cocktail 2 (data not shown). Further, Cocktail 3 was made in which the concentration of Ag85C was increased two times from that of cocktail 1 but the antibody response between cocktail 1 and cocktail 3 was not found to be different (data not shown).

The area under ROC with each antigen was compared with 50% effectiveness. Antigen 85Complex, Ag85C and cocktail of antigens were found to be having *P*-value 0.014, < 0.0001 and <0.0001, respectively (Table 1, Fig. 2). Area under ROC of these antigens was compared among each other and Ag85C was significantly efficient than Ag85complex ($P < 0.0001$) and cocktail ($P = 0.0002$) but there was no difference between Ag85complex and cocktail ($P = 0.055$).

Polymerase chain reaction

PCR targeting IS6110 was positive in 37.50% of patients. When PCR results were compared with ELISA using Ag85C, a very good concordance was observed as 84.84% of the patients positive by PCR were having antibody to Ag85C. Antibody response to Ag85B was noted in 69.69% of patients, to cocktail of antigens in 63.63% of patients and to Ag85complex in 48.48% of patients positive by PCR. However, very poor concordance was noted with CFP-10 followed by Ag85A as only 30.30% and 33.33% of patients positive by PCR were showing antibody reactivity to these antigens, respectively.

Discussion

Secretory antigens of *M. tuberculosis* have been reported to be immunogenic and hence are thought to have diagnostic potential [17]. ESAT-6 and CFP-10 are the secretory antigens encoded by RD1 region genes which are deleted in *M. bovis* BCG but present in *M. tuberculosis* [18]. Humoral response to different components of a major secretory protein Ag85complex in tuberculosis has been reported [19–21]. Hence utility of these antigens in the diagnosis of TB has been envisaged. In the present study, antibody reactivity to secretory antigens CFP-10, Ag85complex and its individual components A, B, C and a mixture of all these antigens along with ESAT-6 was analysed in various types of paediatric TB patients and compared with the antibody response of healthy children and children suffering from other diseases. We observed highest sensitivity and specificity of 89.77% and 92%, respectively, using Ag85C. Positivity of 58.82% was noted in cases under treatment using this antigen; however, only 23.52% of children with other disease were showing reactivity. Sensitivity of 57.95% and specificity of 72% was noted using Ag85complex. However, 35.29% of children undergoing therapy were having antibody to Ag85complex. Similar to our finding in children Rodrigues *et al.* have also noted sensitivity of 72% using Ag85complex in adult TB cases; however, they have noted specificity of 100% and antibody reactivity in 95.8% treated cases [19]. Sensitivity and specificity with Ag85B was noted as 64.77% and 64% respectively, and 47.1% of children undergoing ATT showed positivity. Low reactivity (31.82%) and specificity (24%) were found with Ag85A. Although antibody response to these antigens in childhood TB has not been reported; however, Ag85C 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. On the other hand, antibody to Ag85A and B has been reported to appear in advanced cases of adult TB [24]. Differences noted in our 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 [25]. 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 [13]. Further, sensitivity of 81.4% and sensitivity of 93% were reported by the detection of antibody

to free antigen and immune complexed antigen of ES-31, respectively, in childhood tuberculosis [6].

Sensitivity to CFP-10 was observed to be 29.55%; however, the specificity was noted to be high (80%). No antibody reactivity was observed to this antigen in children undergoing treatment. Similar to our observations, low reactivity (7%) of IgG antibodies against CFP-10 in children has been reported earlier [26]. However, role of CFP-10 in multiepitope ELISA with other two antigens has been reported in adult TB [27].

Taking into view the earlier observations of heterogeneous antibody response of TB patients [9], all the antigens taken in the present study were mixed together and a cocktail 1 was made. Sensitivity and specificity with cocktail 1 was observed to be 67.1% and 80%, respectively. However, reactivity in patients undergoing ATT was found to be quite low at 5.8% and to our surprise it was lower than reactivity noted with Ag85 antigens present in the cocktail. It could be possible that antibody binding to multiepitope is inhibited due to stearic hindrance. On the other hand, use of multiepitope in a serodiagnostic test has been shown to provide incremental increase in sensitivity [6, 27] contrary to our finding with the cocktail antigens. However, using cocktail of antigens all the patients positive by culture and smear examination were showing reactivity and 80% of culture positive individuals were showing antibody to cocktail of antigens.

Antibody response to various antigens was compared with the PCR positivity. We observed very good concordance with the result of PCR and antibody response to Ag85C but little less in case of Ag85B, cocktail of antigens and Ag85complex in a decreasing order. Very low concordance was seen with CFP-10 followed by Ag85A. Earlier we have shown overall 42.2% of positivity by PCR for childhood TB cases [12], which is similar to our findings; however, concordance of PCR with antibody positivity using ESAT-6 was not noted in our previous study.

The present study explores the antibody profiles of paediatric TB patients to various secretory antigens and performance of various diagnostic techniques in these patients. Ag85C was found to be most promising and the best among all the antigens used, having a cross-reactivity lower than other antigens. It was also found superior to PCR and good concordance was also noted with PCR positive patients using this antigen. Although use of polyprotein ELISA was not helpful in increasing either specificity or sensitivity in our study, 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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Conflict of interests

Authors declare that they have no conflict of interests in the present study.

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