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Novel interferon-gamma assays for diagnosing tuberculosis in young children in India

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SUMMARY

SETTING—The tuberculin skin test (TST) and interferon-gamma release assays (IGRAs) are used as supportive evidence to diagnose active tuberculosis (TB). Novel IGRAs could improve diagnosis, but data are lacking in young children.

DESIGN—Children (age 5 years) with suspected TB were prospectively screened at a tertiary hospital in Pune, India; the children underwent TST, and standard (early secretory antigenic target 6 and culture filtrate protein 10) and enhanced (five additional novel antigens) enzyme-linked immunospot (ELISpot) assays.

RESULTS—Of 313 children (median age 30 months) enrolled, 92% had received bacille Calmette-Guérin vaccination, 53% were malnourished and 9% were coinfecting with the human immunodeficiency virus (HIV); 48 (15%) had TB, 128 (41%) did not, and TB could not be ruled out in 137 (44%). The sensitivity of enhanced (45%) and standard (42%) ELISpot assays for diagnosing TB was better than that of TST (20%) ($P = 0.03$); however, enhanced ELISpot was not

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Conflicts of interest: AL holds several patents in the field of T cell-based diagnosis, some of which are licensed to Oxford Immunotec Ltd, Abingdon, UK. The interferon-gamma ELISpot assay developed by AL for diagnosis of tuberculous infection was commercialised by an Oxford University (Oxford, UK) spin-out company (T-SPOT®. TB, Oxford Immunotec), in which Oxford University and AL have royalty entitlements. All other authors declare no conflicts.

more sensitive than the standard ELISpot assay ($P=0.50$). The specificity of enhanced ELISpot, standard ELISpot and TST was respectively 82% (95%CI 74–89), 88% (95%CI 81–94) and 98% (95%CI 93–100). Rv3879c and Rv3615c, previously reported to be promising antigens, failed to improve the diagnostic performance of the ELISpot assay.

CONCLUSION—The TST and the standard and novel ELISpot assays performed poorly in diagnosing active TB among young children in India.

Keywords

TB; children; TST; IGRA; ELISpot; *Rv3615c*; *Rv3879c*; India

MYCOBACTERIUM TUBERCULOSIS is one of the most successful human pathogens. Every year, more than 7.6 million children aged <15 years worldwide become infected with *M. tuberculosis*, 1 million develop active tuberculosis (TB) and nearly 140 000 die.^{1–3} Young children are at high risk of developing severe and often fatal forms of disseminated TB, and childhood TB accounts for nearly 20% of all TB-related deaths in high-burden countries.^{4,5} Rapid and accurate diagnostic tests are therefore critical to prevent morbidity and mortality among children living in TB-prevalent settings. Microbiological confirmation of TB in young children is frequently missed due to the difficulty in obtaining adequate sputum samples, poor yield of clinical specimens due to paucibacillary disease, and the low sensitivity of acid-fast bacilli (AFB) smear microscopy and culture.^{6,7} Establishing a clinical diagnosis of TB is challenging due to its non-specific clinical presentation in young children⁸ and in those with human immunodeficiency virus (HIV) coinfection.⁹

The tuberculin skin test (TST) is widely used to support the diagnosis of TB in young children.¹⁰ However, TST has low sensitivity, and may lead to false-positive results due to its cross-reactivity with the bacille Calmette-Guérin (BCG) vaccine or nontuberculous mycobacteria (NTM), all of which are prevalent in high TB burden settings.¹¹ Interferon-gamma release assays (IGRAs) overcome some of the pitfalls of TST, as they measure ex vivo interferon-gamma (IFN- γ) produced by peripheral blood mononuclear cells (PBMCs) in response to *M. tuberculosis*-specific early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), which are absent in BCG and most NTMs.¹² A study by Liebeschuetz et al. reported a significantly higher sensitivity of the enzyme-linked immunospot (ELI-Spot) based IGRA than TST in diagnosing active TB, particularly among HIV-coinfected and malnourished South African children.¹³ However, subsequent studies have failed to replicate these findings.^{14,15}

The diagnostic performance of the standard ELISpot assay using ESAT-6 and CFP-10 antigens may be further improved by the addition of novel antigens encoded by the regions of difference 1 and 2 (RD-1 and RD-2) of the *M. tuberculosis* genome, gene region-encoding antigens that are absent in BCG and in most NTMs.^{16,17} RD-1 antigens encoded by *Rv3615c* have been shown to be an immunodominant *M. tuberculosis* antigen;¹⁸ Dosanjh et al. reported a modest (4%) increase in the sensitivity of ELISpot assays using novel antigens encoded by *Rv3879c* for the diagnosis of TB among adults in the United Kingdom.¹⁹ However, the generalisability of these data to paediatric populations in high TB burden settings is not known.

We studied the diagnostic utility of enhanced ELISpot assays using five novel antigens encoded by genes from the RD-1 region among young children (age 5 years) with suspected TB in Pune, India.

METHODS

Study participants

We prospectively enrolled young children (age 5 years) presenting with suspected TB from August 2010 to December 2013 by consecutive sampling at the in-patient and out-patient facilities of the Byramjee Jeejeebhoy Government Medical College (BJGMC) and its affiliated clinics in Pune, India. The clinical characteristics of patients enrolled from August 2010 to March 2012 have been published previously by Jain et al.²⁰ Identical protocols were followed in the subsequent time period. Children with suspected TB were classified into the following TB diagnostic categories: definite TB, defined as any clinical sample positive for *M. tuberculosis* using culture or Xpert® MTB/RIF (Cepheid, Sunnyvale, CA, USA); probable TB (Appendix Figure A.1^{*});²⁰ no TB, defined as negative mycobacterial cultures and documented resolution of symptoms without anti-tuberculosis treatment; or possible TB, all other children. To ensure internal validity of our study, TST or IGRAs were not used as criteria for clinical classification of patients.

Written informed consent was obtained from parents or legal guardians on behalf of the children enrolled in our study. The study was approved by the Research Ethics Committees of BJGMC and the National AIDS Research Institute (NARI), Pune, India, and the Johns Hopkins University, Baltimore, MD, USA.

Study procedures

History and physical examination were performed on each participant by trained study physicians. Malnutrition was defined as weight-for-age *Z*-scores (WAZ) <−2 (World Health Organization, version 3.2.2, January 2011). Five tuberculin units (TU) of purified protein derivative (PPD; in 0.1 ml; Radiant Parenterals Ltd, Vaghodia, India) were inoculated intradermally into the forearm during the initial visit and the induration was read at 48–72 h by trained study physicians. As the children were being evaluated for TB, an induration of ≥5 mm was considered as a positive TST.¹⁰ Blood for ELISpot assays was obtained at enrolment and within 24 h of TST placement, which resulted in blood being collected from 2 weeks of symptom onset to within 7 days of starting anti-tuberculosis treatment. For HIV testing, blood was drawn at two separate visits for HIV enzyme-linked immunosorbent assay (ELISA) tests, or HIV DNA polymerase chain reaction (PCR) for children aged <18 months.

ELISpot assays

The standard (using ESAT-6 and CFP-10), and enhanced (using five novel antigens encoded by genes in RD-1 region in addition to ESAT-6 and CFP-10), ELISpot assays were performed at NARI (Table 1). We also included PPD as an antigen in the test wells, although

^{*}The appendix is available in the online version of this article, at <http://www.ingentaconnect.com/content/ijatld/ijatld/2017/00000021/00000004/art00010>

it did not constitute part of any ELISpot assay. Blood samples were transported at 21–25°C and processed within 4–6 h of collection.

Details of the ELISpot assay procedures are described elsewhere.²¹ Briefly, 2.5×10^5 viable PBMCs isolated from heparinised blood were seeded to each well containing a single antigen (10 µg/ml 15mer peptide antigen) coated with IFN- γ (Mabtech AB, Stockholm, Sweden). Wells without any antigen served as negative controls, while those with 5 µg/ml phytohaemagglutinin (ICN Biomedical, Aurora, OH, USA) served as positive controls. All assays were performed in duplicate. Plates were incubated for 16–20 h at 37°C, washed and further incubated for 1 h with anti-IFN- γ monoclonal antibody conjugated to alkaline phosphatase (Mabtech AB). The plates were further treated with the substrate BCIPNBT PLUS (MP Biomedicals, Santa Ana, CA, USA) to visualise the spots. Spot-forming units (SFUs) were counted and scored using an automated ELISpot reader and customised software (AID GmbH, Strassberg, Germany). The test was scored as positive when test wells contained a mean SFU count of at least 5 more SFUs than the mean of the negative control wells if the negative control wells reported 0–5 SFUs, when the test wells contained a mean of at least twice the mean number of SFUs of the negative control wells, if the negative control wells reported 6–10 SFUs, or when the test wells contained a mean of at least 3 times the mean number of SFUs of the negative control wells and the negative control wells reported 11–20 SFUs. Positive control wells containing <20 SFUs or negative control wells containing >20 SFUs per 2.5×10^5 cells constituted an invalid test result. Laboratory technicians performing and reading the assays were blinded to both the TST results and the clinical status of participants.

Statistical analyses

We calculated the sensitivity of the TST and the standard and enhanced ELISpot assays in children with a composite outcome of definite or probable TB (hereafter referred to as active TB), and in those with definite TB. The specificity of the three tests was calculated in children with no TB. Receiver operating characteristic (ROC) curves were constructed for standard ELISpot, enhanced ELISpot and standard ELISpot combined with *Rv3879c*-encoded antigens and standard ELISpot combined with *Rv3615c*-encoded antigens. Area under the curve (AUC) and the associated 95% confidence intervals (CIs) were calculated to evaluate the discriminatory ability of the assays. Children with possible TB and those with missing or invalid ELISpot results were excluded from the analysis. Matched-pairs χ^2 tests for statistical significance were used to account for correlation between the TST, standard ELISpot and enhanced ELISpot tests. The reported *P* values are exact values to account for the small sample size, with $\alpha = 0.05$. Analyses were performed using the ‘diagt’ package in STATA, version 13.0 (Stata Corp, College Station, TX, USA).

RESULTS

Of 431 children with suspected TB screened, 313 were enrolled in the study (Figure 1). The median age of the enrolled children was 30 months (interquartile range [IQR] 13–44); 58% (179/311) were males, 92% (269/294) were BCG-vaccinated and 53% (130/247) were malnourished. Of the 313 children enrolled, 15 (5%) did not have a documented HIV test

result, while 9% (27/298) of the remainder were HIV-coinfected; 15% (48/313) of the children were found to have active TB (definite or probable), 41% (128/313) did not have TB (no TB), while TB could not be ruled out in 44% (137/313) and they were classified as possible TB (Figure 1). History of past exposure to TB was available for 202 children; 46% (92/202) reported contact with a TB case in the previous 2 years. Malnourished, HIV-coinfected and non-BCG-vaccinated children were more likely to have active TB ($P < 0.05$) (Table 2).

Diagnostic performance of the ELISpot assays

Of the 48 children with active TB, 41 (85%) had both TST and ELISpot results available. Respectively 12 (29%) and 10 (24%) had discordant TST and standard or enhanced ELISpot results: 10 with a negative TST but a positive standard ELISpot, 2 with a positive TST but a negative standard ELISpot and 10 with a negative TST but positive enhanced ELISpot results. ELISpot was invalid in 7% (3/46) of children with active TB and 6% (8/127) of those with no TB ($P = 0.95$).

The standard (sensitivity 42%, 95% CI 27–58) and enhanced (sensitivity 45%, 95% CI 31–62) ELISpot assays performed significantly better than the TST (sensitivity 20%, 95% CI 10–35) in detecting active TB ($P = 0.03$ and $P = 0.002$, respectively); however, enhanced ELISpot was not more sensitive than the standard ELISpot assay ($P = 0.50$). In children with definite TB, the sensitivity of enhanced ELISpot, standard ELISpot and TST was respectively 67% (95% CI 38–88), 53% (95% CI 27–79) and 38% (95% CI 15–65); however, this difference did not reach statistical significance (respectively $P = 0.50$ and $P = 0.12$; Table 3). The specificity of TST (98%, 95% CI 93–100) was higher than that of standard ELISpot (88%, 95% CI 81–94) and enhanced ELISpot (82%, 95% CI 74–89) ($P < 0.01$) (Table 3). While there was a trend demonstrating improved sensitivity of the enhanced and standard ELISpot assays compared to the TST in diagnosing active TB among malnourished children and infants, these results were not statistically significant (Table 4). The TST remained specific (97%, 95% CI 93–100) in BCG-vaccinated children (Table 4). Test performance among HIV-coinfected children could not be assessed due to the limited sample size.

Studies in children and adults have demonstrated that the placement of a TST up to 6 weeks before drawing blood does not affect IGRA results.^{22–24} However, one small study in 26 adults suggested that the placement of TST > 3 days before drawing blood could affect IGRA results.²⁵ We therefore analysed the effects of prior TST placement on the ELISpot readouts. Details of the timing of TST placement and blood collection for ELISpot were available for 171 (55%) children: 4 (2%) had definite TB, 14 (8%) had probable TB, 90 (53%) had possible TB and TB was ruled out in 63 (37%). The median time to blood collection following TST placement was 4 days (IQR 2–6); 86 (50%) children had blood collected beyond 3 days of TST placement. However, the median SFU response was not enhanced in any antigen for any group of children—no TB ($n = 63$), possible TB ($n = 90$) or definite/probable TB ($n = 18$)—who had blood collected within 3 days of TST placement vs. those who had blood collected beyond 3 days (Appendix Figure A.2).

Rv3615c and Rv3879c encoded antigens and the ELISpot assay

Given that antigens encoded by *Rv3879c* and *Rv3615c* have been reported to be immunogenic and/or improve sensitivity,^{16,18} we calculated the added diagnostic value of including each antigen to the standard ELISpot assay. The ability of the standard ELISpot assay (AUC 0.52, 95% CI 0.41–0.63) to correctly identify children with active TB failed to improve significantly with the addition of *Rv3615c* (AUC 0.53, 95% CI 0.42–0.63, $P=0.77$) or *Rv3879c* (AUC 0.50, 95% CI 0.39–0.60, $P=0.05$) encoded antigens. Similar results were noted in children with definite TB (AUC of standard ELISpot 0.54, 95% CI 0.37–0.72), and the addition of *Rv3615c* (AUC 0.57, 95% CI 0.40–0.74, $P=0.53$) or *Rv3879c* (AUC 0.52, 95% CI 0.35–0.70, $P=0.07$) encoded antigens failed to improve diagnostic performance (Figure 2). It should be noted that ELISpot using PPD as the antigenic stimulant demonstrated a significantly more robust response among children with active TB (Wilcoxon's sign-rank test $P<0.001$; Figure 3). However, this response did not translate to high discriminatory ability for the PPD-based ELISpot assay (AUC 0.51, 95% CI 0.41–0.62).

DISCUSSION

Our study demonstrates that the TST and ELISpot-based IGRAs performed poorly in diagnosing active TB in young children. While ELISpot assays had better sensitivity than the TST in detecting culture-confirmed or clinically diagnosed TB, adding novel antigens to the standard ELISpot did not improve the diagnostic accuracy. IGRAs are thought to be relatively unaffected by factors such as BCG vaccination, malnutrition and exposure to NTM, which compromise the diagnostic performance of TST, and they have been studied as an alternative to TST for diagnosing TB in children.^{13,26–30} While the study by Liebeschuetz et al. demonstrated that ELISpot was more sensitive than TST for the diagnosis of active TB in children, it did not evaluate specificity.¹³ Moreover, while IGRAs have been found to be more specific than the TST in low TB burden settings,²⁹ this was not the case in studies performed in high TB burden countries.^{31,32} For example, Dogra et al. demonstrated that the TST and QuantiFERON®-TB-Gold In-Tube assay (Qiagen, Hilden, Germany) produced comparable results in BCG-vaccinated children in rural India.²⁶ Meta-analyses have demonstrated that the standard ELISpot assays are not more sensitive or specific than the TST.^{15,33,34} In addition, recent studies have shown that standard ELISpot assays fail to add incremental diagnostic value over the TST in settings where clinical expertise for diagnosing paediatric TB exists.^{14,35} Furthermore, a recent cost analysis suggests that using IGRAs for diagnosis of active TB in a setting such as India results in overtreatment, with tremendous incremental costs.³⁶ Our study results are in line with these previous studies, demonstrating that neither the TST nor ELISpot assays can be used to rule in or rule out TB with reasonable certainty.

While our sensitivity estimates for ELISpot assays are lower than pooled estimates reported in meta-analyses, they are similar to sensitivity estimates from high TB burden settings.^{33,34} It is possible that sensitivity was compromised given that our study population was comprised exclusively of young children, who produce lower IFN- γ responses to *M. tuberculosis* antigens.^{37,38} We also performed analyses to ascertain whether the addition or

combination of specific novel antigens could improve diagnostic accuracy. However, adding *Rv3615c*, recently described by Millington et al. as highly immunodominant,¹⁸ failed to improve the diagnostic performance of the standard ELISpot assay. Similarly, we did not find an improvement in diagnostic accuracy by the inclusion of *Rv3879c*, reported to improve diagnostic accuracy for active TB in adults in a low TB burden setting.¹⁹ Underlying differences between the immune response to TB in adults and in children, and differing levels of background exposure to *M. tuberculosis*, may explain some of these inconsistencies. In the current study, invalid tests were mostly due to failure of the negative control, i.e., > 20 spots in the unstimulated well, indicating non-specific activation of T-cells. Age was not significantly associated with ELISpot validity: the mean age of children with an invalid ELISpot was 34 months compared to a mean age of 30 months in children with valid ELISpots ($P=0.59$). Similarly, age <12 months was not significantly associated with an invalid ELISpot result ($P=0.33$).

Our study has several limitations. First, the sample size was limited as there were few children with definite (culture or Xpert-confirmed) TB. Our study was therefore likely underpowered to detect a true difference in diagnostic performance between the TST and the standard and enhanced ELISpot assays. However, the sensitivity of culture or Xpert is low in children,⁷ and our findings are similar to those reported elsewhere among children evaluated for active TB.^{15,30,33,34} Second, as clinical diagnosis is an imperfect gold standard for active TB in young children, immunological evidence of infection is often used to support a diagnosis of active TB in children. However, to prevent bias, we did not use TST or IGRA results to support a classification under the probable TB category. The true diagnostic performance of the ELISpot assay may therefore be difficult to assess. However, mortality after 2 years' follow-up was lower in patients without TB than in children with definite or probable TB, thus reinforcing our belief in our study's a priori defined diagnostic criteria for TB.²⁰ As blood for the ELISpot assays was drawn a few days after the placement of the TST, we assessed the effect on the timing of the blood drawn in relation to TST placement. The median SFU response to antigens did not differ significantly between those who had blood collected within 3 days of TST placement vs. those who had blood collected after 3 days. Finally, the overall sensitivity of the TST for diagnosing active TB was poor (20%) in this study. While compromised immune response in young children, significant rates of malnourishment and the PPD formulation used locally could explain this finding, paediatricians in this area use the TST cautiously, and a negative result is not used to rule out TB. For example, the decision to start anti-tuberculosis treatment in the current study was solely left to the treating physician, but the majority of TST-negative patients were indeed administered anti-tuberculosis treatment. The low sensitivity of TST may also explain the paradoxically high specificity. For example, nearly half of all the children in the no TB category reported recent exposure to a known case of active TB and may have had latent tuberculous infection. These children may have been ELISpot-positive but false-negative on TST, which could explain the low specificity of the ELISpot assays when used to evaluate active TB in populations in TB-endemic settings with a high prevalence of latent tuberculous infection. However, children in high TB endemic settings are likely to have high levels of exposure to persons with active TB, significantly limiting the use of ELISpot assays for diagnosing active TB disease.

In summary, TST and ELISpot-based IGRAs performed poorly in diagnosing active TB in young children in Pune, India. While ELISpot assays had better sensitivity than the TST in detecting culture-confirmed or clinically diagnosed TB, the addition of novel antigens failed to significantly improve the diagnostic accuracy of the ELISpot assay. Innovative diagnostic approaches for TB are needed in paediatric populations.

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APPENDIX

Figure A1.

TB diagnostic categories (adapted from Jain et al.²⁰). TB = tuberculosis; CSF = cerebrospinal fluid; WBC = white blood cell; CT = computed tomography; MRI = magnetic resonance imaging.

Figure A2.

Median SFU response for children who had blood collected within 3 days of TST placement vs. those who had blood collected beyond 3 days. TB = tuberculosis; SFU = spot-forming unit; PPD = purified protein derivative; ESAT-6 = early secretory antigenic target 6; CFP-10 = culture filtrate protein 10.

References

1. Dodd PJ, Gardiner E, Coghlan R, Seddon JA. Burden of childhood tuberculosis in 22 high-burden countries: a mathematical modelling study. *Lancet Global Health*. 2014; 2:e453–e459. [PubMed: 25103518]
2. Hesselning AC, Cotton MF, Jennings T, et al. High incidence of tuberculosis among HIV-infected infants: evidence from a South African population-based study highlights the need for improved tuberculosis control strategies. *Clin Infect Dis*. 2009; 48:108–114. [PubMed: 19049436]
3. World Health Organization. Global tuberculosis report, 2015. Geneva, Switzerland: WHO; 2015. WHO/HTM/TB/2015.22
4. Marais BJ, Hesselning AC, Gie RP, Schaaf HS, Beyers N. The burden of childhood tuberculosis and the accuracy of community-based surveillance data. *Int J Tuberc Lung Dis*. 2006; 10:259–263. [PubMed: 16562704]
5. Swaminathan S, Rekha B. Pediatric tuberculosis: global overview and challenges. *Clin Infect Dis*. 2010; 50(Suppl 3):S184–S194. [PubMed: 20397947]
6. Cruz AT, Starke JR. Clinical manifestations of tuberculosis in children. *Paediatr Respir Rev*. 2007; 8:107–117. [PubMed: 17574154]
7. Salazar-Austin N, Ordonez AA, Hsu AJ, et al. Extensively drug-resistant tuberculosis in a young child after travel to India. *Lancet Infect Dis*. 2015; 15:1485–1491. [PubMed: 26607130]
8. Perez-Velez CM, Marais BJ. Tuberculosis in children. *N Engl J Med*. 2012; 367:348–361. [PubMed: 22830465]

9. Marais BJ, Graham SM, Cotton MF, Beyers N. Diagnostic and management challenges for childhood tuberculosis in the era of HIV. *J Infect Dis.* 2007; 196(Suppl 1):S76–S85. [PubMed: 17624829]
10. Starke JR, Committee On Infectious Diseases. Interferon-gamma release assays for diagnosis of tuberculosis infection and disease in children. *Pediatrics.* 2014; 134:e1763–1773. [PubMed: 25422024]
11. Watkins RE, Brennan R, Plant AJ. Tuberculin reactivity and the risk of tuberculosis: a review. *Int J Tuberc Lung Dis.* 2000; 4:895–903. [PubMed: 11055755]
12. Lewinsohn DA, Lobato MN, Jereb JA. Interferon-gamma release assays: new diagnostic tests for *Mycobacterium tuberculosis* infection, and their use in children. *Curr Opin Pediatr.* 2010; 22:71–76. [PubMed: 19952926]
13. Liebeschuetz S, Bamber S, Ewer K, Deeks J, Pathan AA, Lalvani A. Diagnosis of tuberculosis in South African children with a T-cell-based assay: a prospective cohort study. *Lancet.* 2004; 364:2196–2203. [PubMed: 15610806]
14. Kampmann B, Whittaker E, Williams A, et al. Interferon-gamma release assays do not identify more children with active tuberculosis than the tuberculin skin test. *Eur Respir J.* 2009; 33:1374–1382. [PubMed: 19196815]
15. Mandalakas AM, Detjen AK, Hesselning AC, Benedetti A, Menzies D. Interferon-gamma release assays and childhood tuberculosis: systematic review and meta-analysis. *Int J Tuberc Lung Dis.* 2011; 15:1018–1032. [PubMed: 21669030]
16. Liu XQ, Dosanjh D, Varia H, et al. Evaluation of T-cell responses to novel RD1 and RD2-encoded *Mycobacterium tuberculosis* gene products for specific detection of human tuberculosis infection. *Infect Immun.* 2004; 72:2574–2581. [PubMed: 15102765]
17. Chiappini E, Della Bella C, Bonsignori F, et al. Potential role of *M. tuberculosis* specific IFN-gamma and IL-2 ELISpot assays in discriminating children with active or latent tuberculosis. *PLOS ONE.* 2012; 7:e46041. [PubMed: 23029377]
18. Millington KA, Fortune SM, Low J, et al. *Rv3615c* is a highly immunodominant RD1 (region of difference 1) dependent secreted antigen specific for *Mycobacterium tuberculosis* infection. *Proc Natl Acad Sci USA.* 2011; 108:5730–5735. [PubMed: 21427227]
19. Dosanjh DP, Hinks TS, Innes JA, et al. Improved diagnostic evaluation of suspected tuberculosis. *Ann Intern Med.* 2008; 148:325–336. [PubMed: 18316751]
20. Jain SK, Ordonez A, Kinikar A, et al. Pediatric tuberculosis in young children in India: a prospective study. *BioMed Res Int.* 2013; 2013:783698. [PubMed: 24386640]
21. Lalvani A, Pathan AA, McShane H, et al. Rapid detection of *Mycobacterium tuberculosis* infection by enumeration of antigen-specific T cells. *Am J Respir Crit Care Med.* 2001; 163:824–828. [PubMed: 11282752]
22. Naseer A, Naqvi S, Kampmann B. Evidence for boosting *Mycobacterium tuberculosis*-specific IFN-gamma responses at 6 weeks following tuberculin skin testing. *Eur Respir J.* 2007; 29:1282–1283. [PubMed: 17540791]
23. Richeldi L, Bergamini BM, Vaienti F. Prior tuberculin skin testing does not boost QuantiFERON-TB results in paediatric contacts. *Eur Respir J.* 2008; 32:524–525. [PubMed: 18669795]
24. Richeldi L, Ewer K, Losi M, Roversi P, Fabbri LM, Lalvani A. Repeated tuberculin testing does not induce false positive ELISpot results. *Thorax.* 2006; 61:180.
25. van Zyl-Smit RN, Pai M, Peprah K, et al. Within-subject variability and boosting of T-cell interferon-gamma responses after tuberculin skin testing. *Am J Respir Crit Care Med.* 2009; 180:49–58. [PubMed: 19342414]
26. Dogra S, Narang P, Mendiratta DK, et al. Comparison of a whole blood interferon-gamma assay with tuberculin skin testing for the detection of tuberculosis infection in hospitalized children in rural India. *J Infect.* 2007; 54:267–276. [PubMed: 16733068]
27. Moyo S, Isaacs F, Gelderbloem S, et al. Tuberculin skin test and QuantiFERON® assay in young children investigated for tuberculosis in South Africa. *Int J Tuberc Lung Dis.* 2011; 15:1176–1181. i. [PubMed: 21943841]

28. Schopfer K, Rieder HL, Bodmer T, et al. The sensitivity of an interferon-gamma release assay in microbiologically confirmed pediatric tuberculosis. *Eur J Pediatr*. 2014; 173:331–336. [PubMed: 24065457]
29. Detjen AK, Keil T, Roll S, et al. Interferon-gamma release assays improve the diagnosis of tuberculosis and nontuberculous mycobacterial disease in children in a country with a low incidence of tuberculosis. *Clin Infect Dis*. 2007; 45:322–328. [PubMed: 17599309]
30. Bamford AR, Crook AM, Clark JE, et al. Comparison of interferon-gamma release assays and tuberculin skin test in predicting active tuberculosis (TB) in children in the UK: a paediatric TB network study. *Arch Dis Child*. 2010; 95:180–186. [PubMed: 19815937]
31. Ling DI, Pai M, Davids V, et al. Are interferon-gamma release assays useful for diagnosing active tuberculosis in a high-burden setting? *Eur Respir J*. 2011; 38:649–656. [PubMed: 21349910]
32. Metcalfe JZ, Everett CK, Steingart KR, et al. Interferon-gamma release assays for active pulmonary tuberculosis diagnosis in adults in low- and middle-income countries: systematic review and meta-analysis. *J Infect Dis*. 2011; 204(Suppl 4):S1120–S1129. [PubMed: 21996694]
33. Machingaidze S, Wiysonge CS, Gonzalez-Angulo Y, et al. The utility of an interferon gamma release assay for diagnosis of latent tuberculosis infection and disease in children: a systematic review and meta-analysis. *Pediatr Infect Dis J*. 2011; 30:694–700. [PubMed: 21427627]
34. Sun L, Xiao J, Miao Q, et al. Interferon gamma release assay in diagnosis of pediatric tuberculosis: a meta-analysis. *FEMS Immunol Med Microbiol*. 2011; 63:165–173. [PubMed: 22077219]
35. Ling DI, Nicol MP, Pai M, Pienaar S, Dendukuri N, Zar HJ. Incremental value of T-SPOT. *TB* for diagnosis of active pulmonary tuberculosis in children in a high-burden setting: a multivariable analysis. *Thorax*. 2013; 68:860–866. [PubMed: 23674550]
36. Little KM, Pai M, Dowdy DW. Costs and consequences of using interferon-gamma release assays for the diagnosis of active tuberculosis in India. *PLOS ONE*. 2014; 10:e0124525. [PubMed: 25918999]
37. Kampmann B, Tena-Coki G, Anderson S. Blood tests for diagnosis of tuberculosis. *Lancet*. 2006; 368:282. author reply -3.
38. Lewinsohn DA, Gennaro ML, Scholvinck L, Lewinsohn DM. Tuberculosis immunology in children: diagnostic and therapeutic challenges and opportunities. *Int J Tuberc Lung Dis*. 2004; 8:658–674. [PubMed: 15137550]

Figure 1.

Consort diagram. Of the 302 (96%) children who had blood available for ELISpot assays,* 30 (9%) had invalid ELISpot results; 303 (97%) TSTs were placed and read. ‘Definite TB’ included children with any clinical specimen positive for mycobacterial culture. ‘Probable TB’ included children without microbiological confirmation but with a clinical picture suggestive of active TB. ‘No TB’ included children with negative mycobacterial cultures and complete resolution of symptoms without TB treatment. ‘Possible TB’ comprised all other children in whom TB could be neither confirmed nor excluded. * ELISpot includes standard and enhanced IGRA results. TB = tuberculosis; TST = tuberculin skin test; ELISpot = enzyme linked immunospot-based assay; IGRA = interferon-gamma release assay.

Figure 2.

A) ROC for standard ELISpot, enhanced ELISpot, standard ELISpot combined with *Rv3615c* antigens and standard ELISpot combined with *Rv3879c* antigens for the diagnosis of definite TB. AUCs for standard ELISpot = 0.54 (95% CI 0.37–0.72, P = reference), enhanced ELISpot = 0.52 (95% CI 0.35–0.69, P = 0.67), standard ELISpot with *Rv3615c* = 0.57 (95% CI 0.40–0.74, P = 0.53), standard ELISpot with *Rv3879c* = 0.52 (95% CI 0.35–0.70, P = 0.07). P values are for differences between AUCs with standard ELISpot as reference. **B)** ROC for standard ELISpot, enhanced ELISpot, standard ELISpot combined with *Rv3615c* antigens and standard ELISpot combined with *Rv3879c* antigens for the diagnosis of active TB. AUCs for standard ELISpot = 0.52 (95% CI 0.41–0.63, P = reference), enhanced ELISpot = 0.48 (95% CI 0.38–0.59, P = 0.18), standard ELISpot with *Rv3615c* = 0.53 (95% CI 0.42–0.63, P = 0.77), standard ELISpot with *Rv3879c* = 0.50 (95% CI 0.39–0.60, P = 0.05). P values are for differences between AUCs with standard ELISpot as reference. ELISpot = enzyme linked immunospot-based assay; ROC = receiver operating characteristic; TB = tuberculosis; AUC = area under the curve; CI = confidence interval.

This image can be viewed online in colour at <http://www.ingentaconnect.com/content/ijatld/ijatld/2017/00000021/00000004/art00010>

Figure 3.

A) ELISpot assay median SFUs in response to recombinant *Mycobacterium tuberculosis* antigens among children with and those without active TB. Median SFUs in response to PPD were significantly higher than those in response to ESAT-6, CFP-10, *Rv3878c*, *Rv3878*, *Rv3873*, *Rv2654* and *Rv3615c* antigens ($P < 0.001$) among children with and those without active TB. **B)** ELISpot assay median SFUs in response to recombinant *M. tuberculosis* antigens among children in the no TB category and those with definite TB. Median SFUs in response to PPD were significantly higher than those in response to ESAT-6, *Rv3878c*, *Rv3878*, *Rv3873*, *Rv2654* and *Rv3615c* antigens ($P < 0.01$), but not CFP-10 ($P = 0.10$) among children with definite TB. TB = tuberculosis; SFU = spot-forming unit; PPD = purified protein derivative; ESAT-6 = early secretory antigenic target 6; CFP-10 = culture filtrate protein 10. This image can be viewed online in colour at <http://www.ingentaconnect.com/content/ijatld/ijatld/2017/00000021/00000004/art00010>

Table 1

Recombinant mycobacterial antigens used in the ELISpot assays *

Gene number	Product	Comments
<i>Rv3875</i>	ESAT-6	A core mycobacterial gene that is conserved in mycobacterial strains but not expressed by <i>Mycobacterium bovis</i> strains used in BCG vaccines. Potential vaccine candidate
<i>Rv3874</i>	CFP-10	A core mycobacterial gene conserved in mycobacterial strains but not expressed by <i>M. bovis</i> strains used in BCG vaccines
<i>Rv3879c</i>	ESX-1 secretion-associated protein EspK	ESX-1 secretion-associated protein. May improve diagnostic performance of standard ELISpot assays among adults with active tuberculosis ^{16,19}
<i>Rv3615c</i>	ESX-1 secretion-associated protein EspC	Potential vaccine candidate. Likely to be a key target of cellular immunity against <i>M. tuberculosis</i> ¹⁸
<i>Rv3878</i>	ESX-1 secretion-associated protein EspJ	Predicted to be an outer membrane protein
<i>Rv3873</i>	PPE family protein PPE68	Potential vaccine candidate
<i>Rv2654</i>	Possible <i>phiRv2</i> prophage protein	Hypothetical alanine-rich protein and possibly a <i>phiRv2</i> phage protein

* Standard ELISpot contains ESAT-6 and CFP-10 while enhanced ELISpot contains all other antigens in addition to ESAT-6 and CFP-10. ELISpot = enzyme-linked immunospot; ESAT-6 = early secretory antigenic target 6; BCG = bacille Calmette-Guérin; CFP-10 = culture filtrate protein 10; ESX-1 = ESAT-6 secretion system 1; PPE = proline-proline-glutamic acid.

Table 2

Baseline demographic characteristics of enrolled children by TB case definitions

	All (n = 313) n/N (%) [*]	Definite TB (n = 17, 5%) n/N (%) [*]	Probable TB (n = 31, 10%) n/N (%) [*]	Possible TB (n = 137, 44%) n/N (%) [*]	No TB (n = 128, 41%) n/N (%) [*]	P value [†]
Age, months, median [IQR]	30 [13–44]	11 [6–36]	25 [12–48]	30 [12–43]	31 [18–45]	0.23 [‡]
Male sex	179/311 (58)	8/17 (47)	20/30 (67)	75/136 (55)	76/128 (59)	0.53
BCG scar	269/294 (92)	13/16 (81)	21/26 (81)	117/128 (91)	118/124 (95)	0.03 ^{§¶}
HIV-coinfected	27/298 (9)	1/16 (6)	6/26 (23)	15/131 (11)	5/125 (4)	0.01 ^{§¶}
Exposed to a TB case in the past 2 years	92/202 (46)	4/7 (57)	3/15 (20)	52/109 (48)	33/71 (46)	0.20
Malnutrition [#]	130/247 (53)	5/9 (55)	9/23 (39)	76/119 (64)	40/96 (42)	0.005 ^{§¶}

^{*} All percentages rounded to the nearest whole number.[†] Significance determined at the 0.05 level.[‡] Kruskal-Wallis χ^2 test.[§] Fisher's exact test.[¶] Statistically significant.[#] Defined as WHO weight-for-age Z-scores (WAZ) < -2.

TB = tuberculosis; IQR = interquartile range; BCG = bacille Calmette-Guérin; HIV = human immunodeficiency virus; WHO = World Health Organization.

Table 3

Test performance for the diagnosis of active TB

	TST <i>n</i> (%) [*] (95% CI)	Standard ELISpot [†] <i>n</i> (%) [*] (95% CI)	Enhanced ELISpot [†] <i>n</i> (%) [*] (95% CI)
Sensitivity			
Definite/probable TB [‡]	9/45 (20) (10–35)	18/43 (42) (27–58)	20/43 (45) (31–62)
Definite TB [§]	6/16 (38) (15–65)	8/15 (53) (27–79)	10/15 (67) (38–88)
Specificity			
No TB [¶]	124/127 (98) (93–100)	105/119 (88) (81–94)	98/119 (82) (74–89)
Predictive values [#]			
PPV	9/12 (75) (43–95)	18/32 (56) (38–74)	20/41 (49) (33–65)
NPV	124/160 (78) (70–84)	105/130 (81) (73–87)	98/121 (81) (73–88)

^{*} All percentages rounded to the nearest whole number.

[†] ELISpot was invalid in 7% (3/46) of children with TB and 6% (8/127) of those with no TB.

[‡] Proportion of children testing positive for a given test among those with culture-confirmed or clinically diagnosed TB.

[§] Proportion of children testing positive for a given test among those with culture-confirmed TB only.

[¶] Proportion of children testing negative for a given test among those in whom TB was excluded.

[#] Predictive values for definite/probable TB at baseline evaluation.

TB = tuberculosis; TST = tuberculin skin test; CI = confidence interval; ELISpot = enzyme linked immunospot based assay; PPV = positive predictive value; NPV = negative predictive value.

Table 4

Effect of malnourishment, BCG vaccination and very young age on test performance for the diagnosis of active tuberculosis *

	TST n (%)[†] (95% CI)	Standard ELISpot n (%)[†] (95% CI)	Enhanced ELISpot n (%)[†] (95% CI)
Malnourished			
Sensitivity	1/12 (8) (0–39)	4/11 (36) (11–70)	5/11 (46) (17–77)
Specificity	40/40 (100) (91–100)	31/38 (82) (66–92)	31/38 (82) (66–92)
BCG-vaccinated			
Sensitivity	7/33 (21) (9–39)	10/30 (33) (17–53)	11/30 (37) (20–56)
Specificity	114/117 (97) (93–100)	97/109 (89) (82–94)	91/109 (84) (75–90)
Age <1 year			
Sensitivity	2/12 (17) (2–48)	6/12 (50) (21–79)	6/12 (50) (21–79)
Specificity	15/15 (100) (78–100)	14/15 (93) (68–100)	13/15 (87) (60–98)

* Diagnostic performance in HIV-coinfected children could not be calculated due to the very small sample size.

[†] All percentages rounded to the nearest whole number.

BCG = bacille Calmette-Guérin; TST = tuberculin skin test; CI = confidence interval; ELISpot = enzyme linked immunospot based assay; HIV = human immunodeficiency virus.