Use of tuberculin skin test, IFN-γ release assays and IFN-γ-induced protein-10 to identify children with TB infection

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ABSTRACT: Current tests of tuberculosis (TB) infection (tuberculin skin test (TST), interferon (IFN)-γ-release assays (IGRAs) and IFN-γ-induced protein (IP)-10) have limitations and their value when used consecutively to identify infected children has not been explored.

This study describes TST, IGRA and IP-10 responses in children in contact with adults with TB, the agreement of the tests and whether using multiple tests indentifies more infected children. 330 children (aged 1–15 yrs) in contact with adults with pulmonary TB and 156 controls were studied in Ethiopia.

Children exposed to adults with high bacilli grades in sputum were more likely to have positive TST, IFN-γ and IP-10 than controls. The agreement of positive tests was directly associated with the sputum bacilli grades (p<0.001 for all). The agreement of negative tests was higher in control children. The consecutive use of the tests increased the number of children classified as having at least one positive test.

Using three tests increases the number of children classified as infected. This increase is associated with the bacilli load of the adults. Using only one test may underestimate the proportion of infected children, but the interpretation of the data is difficult due to the lack of reference standards.

KEYWORDS: Children, diagnosis, interferon-γ, interferon-γ-induced protein-10, latent tuberculosis, tuberculin skin test

Children in contact with adults with smear-positive pulmonary tuberculosis (PTB) are at high risk of infection. Although this risk is associated with the closeness of contact and the concentration of bacilli in the sputum of the adult index case [1], their investigation is often unrewarding because, besides the century-old tuberculin skin test (TST), there are very few tests to identify tuberculosis (TB) infections in children. The interferon (IFN)-γ-release assays (IGRAs), which are said to be more specific than the TST as they do not cross-react with most nontuberculous mycobacteria or the antigens present in the bacille Calmette–Guérin (BCG) vaccine, represented a step change in the diagnosis of infection in recent decades. Their sensitivity, however, can be compromised in young children, in those with severe malnutrition or HIV co-infection [2] and a negative test does not exclude infection. Most recently, a further marker, the IFN-γ-induced protein (IP)-10, has been suggested as a potential test to identify TB infections. The advantage of IP-10 is that it is produced in high levels following *Mycobacterium tuberculosis* antigen-specific stimulation in both active and latent TB cases, demonstrating its potential as a biomarker for *M. tuberculosis* in children [3]. The performance of IP-10 is also said to be independent of age and less compromised in individuals co-infected with HIV [4], but the test is newer and is less standardised than the TST and IGRAs.

There are limited data on the additional value of combining the TST, IGRAs and IP-10 test results to enhance the identification of infected children and whether IP-10 response patterns vary according to the number of bacilli contained in the sputum of the adult case, as previously demonstrated for the TST and IGRAs.

We describe here whether the concomitant use of TST, IGRAs and IP-10 in children in contact with adults with smear-positive PTB increases the number of children that could be labelled as

infected, and whether these results vary with the concentration of bacilli in the adults' sputum.

MATERIALS AND METHODS

This was a cross-sectional study of 1–15-yr-old children in contact with adults with smear-positive PTB and community controls without known contact with TB. The study was conducted in Hawassa zone, in the Southern Region of Ethiopia. Adults with a history of cough >2 weeks who had a diagnosis of sputum smear-positive PTB were identified consecutively in Hawassa and Bushullo Major Health Centres and Hawassa Referral Hospital. Patients who had children and resided within a 20-km radius of Hawassa were invited to participate and visited at home. Community controls were defined as apparently healthy children without known contact with adults with PTB and were selected from Hawassa and surrounding rural areas after preparing a list of the villages within a 20-km radius of Hawassa. Villages were selected at random and one household was selected from each village by spinning a pen somewhere between the centre and the edge of the village. One child aged 1–15 yrs identified at random from the selected household was invited to participate.

All participant children were applied a TST using 2 U of purified protein derivative (PPD RT 23; Statens Serum Institut, Copenhagen, Denmark) using the Mantoux method and indurations were measured using the palpation method 48–72 h later. TST results were graded as negative (<5 mm), intermediate (≥5 and <10 mm) and positive (≥10 mm). Blood samples for IGRAs were collected using the QuantiFERON-TB Gold In-Tube (QFT-IT) test (Cellestis, Victoria, Australia) following the manufacturer's instructions. Supernatant plasma was harvested from the QFT-IT tubes after centrifugation and stored at -70°C. IFN-γ was measured using the QFT-IT ELISA in a Bio-Rad (Hemel Hempstead, UK) plate reader (model 550), read at 450 nm and classified as positive, negative or indeterminate using the manufacturer's software. IP-10 concentrations were measured in the same supernatants using a Human IP-10 ELISA Construction Kit (Antigenix America Inc., New York, NY, USA) and classified as positive or negative according to a receiver operating curve, as previously described [5]. HIV status was established using two blood-based ELISA methods.

The number of bacilli in the adults sputum was graded as ''scanty'', +, ++ or +++ and used to stratify the proportion of children with positive TST, IFN-γ or IP-10. The added value of IFN-γ and IP-10 were calculated for children with negative TST or both negative TST and IFN-γ to describe whether using multiple tests would increase the proportion of children with at least one positive test.

The study protocol was approved by the Health Bureau of the Southern Region, Ethiopia, the Research Ethics Committees of the Liverpool School of Tropical Medicine and Hawassa University, and the Ethiopian Sciences and Technology Commission. Children were enrolled after obtaining informed parental consent. The study is registered in the Clinicaltrials.gov clinical trials register (number NCT00456469).

RESULTS

A total of 486 children were enrolled. Of these, 330 (median (range) age 8 (1–15) yrs) were contacts and 156 (median (range)

age 6 (1–15) yrs) were community controls, with similar proportions of contacts (n=167, 51%) and controls (n=79, 51%) being male. The sputum smears of the adults of 15 (4.5%) children were graded +++; 109 (33%) were graded ++; 188 (57%) were graded +; and 18 (5.5%) were graded as having ''scanty'' bacilli. 17 (3.5%) children did not have TST results and 28 (6%) did not have IFN-γ or IP-10 results.

The proportion of children with positive IFN-γ or positive IP-10 increased with increasing adult sputum grades, ranging from 17.6% and 23.5% in contacts of adults with scanty grades, to 57% and 50% in contacts with adults with +++ grades, respectively (Chi-squared for trend p<0.001 for both), as shown in table 1. The proportion of children with a positive TST (86.7%) was also highest among children in contact with adults with +++ grades. However, children in contact with adults with scanty bacilli also had a high proportion of positive TST results. In contrast, the proportion of children with positive TST, IFN-γ and IP-10 was low in controls (12.8%, 13.1% and 5.8%, respectively). Figure 1 describes the percentage of positive test results for each of the three tests by the adults' smear microscopy grades.

Figure 2 describes the concordance of TST, IFN-γ and IP-10. The proportion of children with positive TST and IFN-γ or with positive TST and IP-10 increased with increasing sputum grades in the adults; with lower concordance among controls and higher concordance in children in contact with adults with high sputum grades (+++). Similarly, the negative concordance of TST, IFN-γ and IP-10 was higher in controls than among children in contact with adults with ++/+++ sputum grades. Concordance between IFN-γ and IP-10 did not vary with the adults' sputum grade.

435 (282 contact and 153 control) children had results for the three tests. After exclusion of intermediate TST and indeterminate IFN-γ results, 313 children had a full set of interpretable

| TABLE 1 | Tuberculin skin test (TST), interferon (IFN)-γ and IFN-γ-induced protein (IP)-10 results in children by sputum grade of the index case |

	Bacilli count of the adult				Controls	Total
	+++	++	+	Scanty		
Subjects	15	109	188	18	156	486
TST						
Positive	13 (86.7)	46 (43)	95 (54.6)	14 (82.4)	20 (12.8)	469
Intermediate	0	19 (17.8)	28 (16.1)	1 (5.9)	15 (9.6)	
Negative	2 (13.3)	42 (39.3)	51 (29.3)	2 (11.8)	121 (77.6)	
IFN-γ						
Positive	8 (57.1)	53 (50.5)	74 (43.8)	3 (17.6)	20 (13.1)	458
Indeterminate	5 (35.7)	13 (12.4)	24 (14.2)	10 (58.8)	25 (16.3)	
Negative	1 (7.1)	39 (37.1)	71 (42)	4 (23.5)	108 (70.6)	
IP-10#						
Positive	7 (50)	60 (59.4)	96 (55.8)	4 (23.5)	9 (5.8)	458
Negative	7 (50)	41 (40.6)	76 (44.2)	13 (76.5)	145 (94.2)	

Data are presented as n or n (%). #: IP-10 positive grading ≥3,022 pg·mL⁻¹, as calculated by the receiver operating characteristic curve.
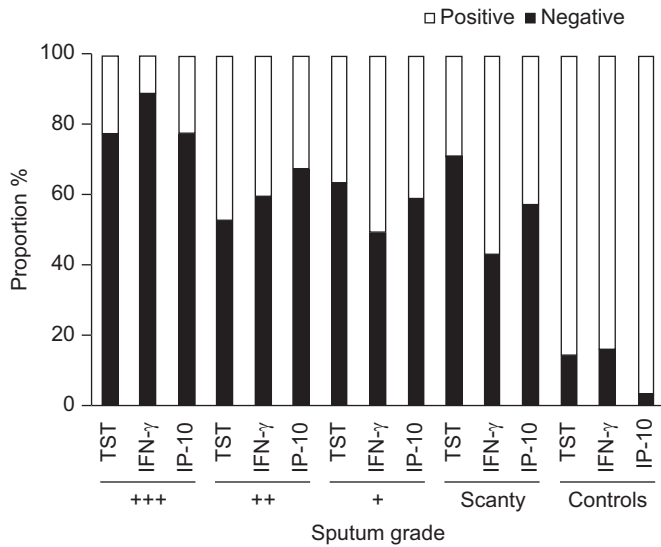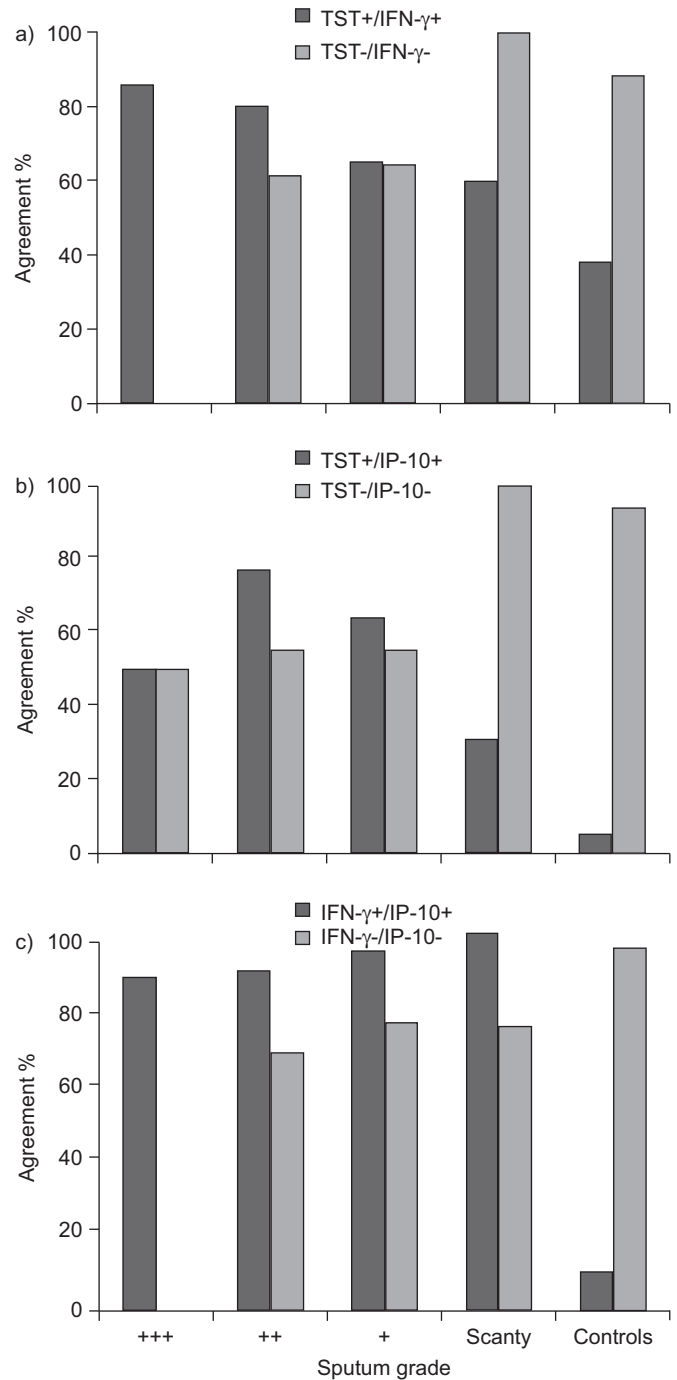
FIGURE 1. Proportion of children with positive tuberculin skin test (TST), interferon (IFN)-γ and IFN-γ-induced protein (IP)-10 results by sputum grade of the index case.

results. These included nine children in contact with adults with +++, 74 with ++, 109 with +, seven with scanty sputum grades and 114 controls. Figure 3 describes the proportion of children that would be classified as infected by using the TST result alone, by adding the IFN-γ result to TST-negative children and by adding the IP-10 result to TST- and IFN-γ-negative children. The results are presented stratified by the adults' sputum grades. All children exposed to adults with +++ grading were positive by TST or IFN-γ and thus IP-10 did not add further positive results. A high proportion of children exposed to adults with + and ++ sputum grades had positive TST or IFN-γ results (72% and 70%, respectively). These percentages increased to 75% and 78%, respectively, by adding IP-10 as a third marker. Of note, a small but significant number of community controls had negative TST but positive IFN-γ (12 out of 114 children) or negative TST and IFN-γ but positive IP-10 (three out of 114 children).

The same proportion of contacts and controls received BCG (249 (74.3%) out of 335 *versus* 120 (76.9%) out of 156, p>0.5). Although contacts were more likely to have positive TST and IFN-γ than controls, the responses within the group were similar regardless of their BCG status (p>0.5 for both TST and IFN-γ among children with and without BCG). The number of children infected with HIV was higher in contact children than in control children (27 (10%) out of 258 and three (2%) out of 156 children tested, respectively; p<0.01). The low number of HIV-infected children, however, precludes further analysis of TST and IFN-γ results stratified by HIV (table 2).

DISCUSSION

Children in contact with adults with smear-positive PTB are at high risk of infection and disease progression. The World Health Organization (WHO) recommends initiating isoniazid prophylaxis for young children in close contact with an adult with TB without investigating whether the child is infected. However, parents are frequently reluctant to provide prophylaxis and

FIGURE 2. Concordance between tuberculin skin test (TST), interferon (IFN)-γ and IFN-γ induced protein (IP)-10 results by sputum grade of the index case. a) TST *versus* IFN-γ; b) TST *versus* IP-10; c) IFN-γ *versus* IP-10.

often abandon the 6-month recommended course [6–9]. It might be that identifying infected children and targeting prophylaxis to high-risk groups could be more acceptable to parents, but the poor sensitivity of TST, its cross-reactions with BCG, and its unreliability in children with malnutrition, immunosupression and severe infections [10] limit the applicability of this approach. The limitations of IGRAs are also becoming apparent with their widespread use in recent years. TB is most endemic in locations where accessibility to diagnostics is a major barrier and thus the
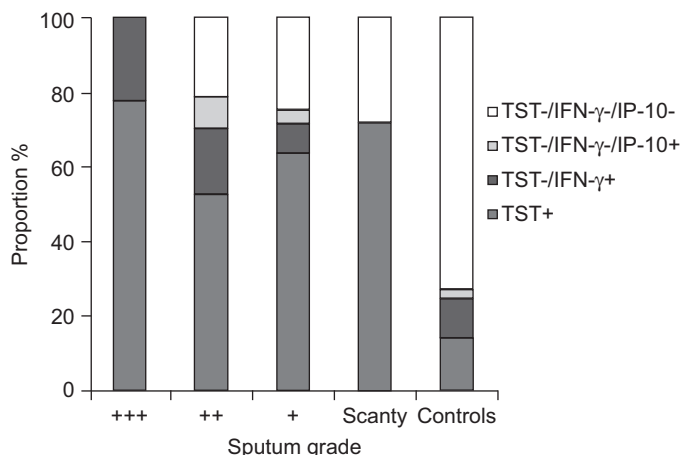
FIGURE 3. Proportion of children with positive tuberculin skin test (TST); positive interferon (IFN)-γ and negative TST; and positive IFN-γ-induced protein (IP)-10 and negative TST and IFN-γ by sputum grade of the index case.

applicability of these tests is limited by their costs, availability and laboratory requirements. Furthermore, their well-documented phenomenon of conversions (from negative to positive) and reversions (from positive to negative) is frequent but poorly understood [11]. False-negative responses have also been reported in very young children and those infected with HIV [12], although they occur at a lower frequency than with the TST. In addition, IGRAs have the logistical difficulty of collecting blood from young children, test processing and procurement, which results in a sizable number of children not having test results available [12]. A recent policy statement from WHO indicated that there was insufficient data on the performance of IGRAs in low- and middle-income countries, and that given the higher costs the use of IGRAs in these settings is not recommended [13]. IGRAs, however, have high specificity and their use in tandem with TST may have added diagnostic value [2, 14]. Several screening strategies to identify individuals with TB infection are emerging internationally. These include using either TST or IGRAs alone, using both tests independently or using them consecutively, usually the TST followed by IGRAs. The latter approach is considered the most cost-effective scheme

in some settings, although there is only limited objective evidence to date [15].

IP-10, an additional marker recently reported to identify TB infections [16], is expressed in larger quantities than IFN-γ, making it easier to measure in blood. HIV-infected individuals are able to express IP-10 [4], which is probably due to some IP-10-expressing cells being spared by the HIV infection. Although its performance is less well documented than TST and IFN-γ testing, it has been suggested to have added value in identifying infections when used together with IGRAs [17, 18].

The risk of infection in most groups is associated to the number of bacilli in the sputum of the adult, as the three markers were increasingly positive with increasing sputum grades. The use of three markers increased the number of individuals with at least one positive test, reaching 100% in children in contact with adults with +++ grading. The agreement of the tests varied with the sputum smear grades, with a higher proportion of tests being simultaneously positive among children exposed to adults with a +++ grading and a lower concordance among contact children who had low risk of infection. Unfortunately, the number of children in the +++ category was small and the study is underpowered to detect an added value in these children, as this is the group where the previous tests are more likely to be positive, thus leaving a small margin for added value. If all tests are interpreted as true positives, a very high proportion of children in contact with adults with TB would be infected. The use of the smear grades, as a proxy of the risk of infection, suggests that the test responses may be true positives; however, this interpretation is problematic as the evaluation of the tests is hampered by the lack of a suitable reference standard and without this reference standard it is impossible to differentiate correct from incorrect test results. Furthermore, the increasing number of controls with a single positive result might reflect a low false-positive test rate, which accumulates when using several tests consecutively. Thus, our results may indicate that a significant proportion of children with asymptomatic infections may be missed by the use of a single test and/or that a significant number of children at low risk of infection may have at least one positive test and that the simultaneous use of tests should be interpreted with caution in low-risk populations.

TABLE 2	Tuberculin skin test (TST) and interferon (IFN)-γ results in children by bacille Calmette–Guérin (BCG) and HIV status							
	Contacts				Controls			
	BCG+	BCG-	HIV+	HIV-	BCG+	BCG-	HIV+	HIV-
TST								
Positive	135 (56)	29 (47.5)	12 (48)	123 (56.7)	16 (13.3)	2 (5.9)	0	20 (13.1)
Intermediate	38 (15.8)	9 (14.8)	5 (20)	24 (11)	12 (1)	3 (8.8)	0	15 (9.8)
Negative	68 (28.2)	23 (37.7)	8 (32)	70 (32.3)	92 (76.6)	29 (85.3)	3 (100)	118 (77.1)
IFN-γ								
Positive	105 (46)	24 (36.4)	10 (41.6)	93 (41.5)	13 (10.9)	6 (18.2)	0	20 (13.3)
Indeterminate	32 (14)	20 (30.3)	4 (16.7)	44 (19.6)	23 (19.3)	2 (6)	0	25 (16.7)
Negative	91 (40)	22 (33.3)	10 (41.6)	87 (38.8)	83 (69.8)	25 (75.8)	3 (100)	105 (70)

Data are presented as n (%).

Although the interpretation of the data remains problematic, the TST in combination with IFN-γ and IP-10 testing increases the proportion of children with one or more positive test results. This increase is in proportion with the bacilli load in the adults' sputum smear. A high proportion of contacts had at least one marker of infection, suggesting that the proportion of children infected after exposure to adults with PTB may be underestimated by using a single test for diagnosis.

CLINICAL TRIAL
This study is registered at ClinicalTrials.gov with identifier number NCT00456469.

STATEMENT OF INTEREST
None declared.

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