Assessment of the novel T-cell activation marker–tuberculosis assay for diagnosis of active tuberculosis in children: a prospective proof-of-concept study

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Summary

Background **The diagnosis of paediatric tuberculosis is complicated by non-specific symptoms, difficult specimen collection, and the paucibacillary nature of the disease. We assessed the accuracy of a novel immunodiagnostic T-cell activation marker–tuberculosis (TAM-TB) assay in a proof-of-concept study to identify children with active tuberculosis.**

Methods **Children with symptoms that suggested tuberculosis were prospectively recruited at the NIMR-Mbeya Medical Research Center in Mbeya, and the Ifakara Health Institute in Bagamoyo, Tanzania, between May 10, 2011, and Sept 4, 2012. Sputum and peripheral blood mononuclear cells were obtained for *Mycobacterium tuberculosis* culture and performance assessment of the TAM-TB assay. The children were assigned to standardised clinical case classifications based on microbiological and clinical findings.**

Findings **Among 290 children screened, we selected a subgroup of 130 to ensure testing of at least 20 with culture-confirmed tuberculosis. 17 of 130 children were excluded because of inconclusive TAM-TB assay results. The TAM-TB assay enabled detection of 15 of 18 culture-confirmed cases (sensitivity 83·3%, 95% CI 58·6–96·4). Specificity was 96·8% (95% CI 89·0–99·6) in the cases that were classified as not tuberculosis (n=63), with little effect from latent tuberculosis infection. The TAM-TB assay identified five additional patients with highly probable or probable tuberculosis, in whom *M tuberculosis* was not isolated. The median time to diagnosis was 19·5 days (IQR 14-45) for culture.**

Interpretation **The sputum-independent TAM-TB assay is a rapid and accurate blood test that has the potential to improve the diagnosis of active tuberculosis in children.**

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Introduction

Tuberculosis in children is a serious public health problem. Recent estimates of the tuberculosis disease burden in children, based on the results of a systematic literature review and mathematical modelling, suggest that about 1 million children developed tuberculosis worldwide in 2010, including 280 000 incident cases in the African region.[1] Surveillance data for children remain imprecise because paediatric tuberculosis is often either underdiagnosed or overdiagnosed in high-burden countries.[2–4] The lack of accurate and rapid diagnostic methods contributes to tuberculosis morbidity and mortality in children and hampers the assessment of new drugs and vaccines in paediatric populations.[4]

Diagnosis of active tuberculosis in children poses a major challenge. Clinical symptoms of tuberculosis in children are often non-specific and resemble those of common paediatric illnesses, including pneumonia and malnutrition. Adequate respiratory specimens are difficult to obtain for bacterial confirmation, particularly in very young children who are unable to expectorate and in whom diagnostic yields are poor because of the paucibacillary nature of the disease.[2,5] Hence, the diagnosis is routinely made on the basis of a combination of clinical features, contact history, chest radiography, and tuberculin skin test, and often with scoring charts that have poor diagnostic accuracy.[6,7]

The Xpert MTB/RIF assay enables timely, sensitive, and specific molecular detection of pulmonary tuberculosis and rifampicin resistance in adults,[8] but its value in young children is greatly reduced. In a recent meta-analysis, the calculated pooled sensitivity was 66% for Xpert MTB/RIF against culture in expectorated or induced sputa, or gastric lavage specimens from children with suspected tuberculosis.[9] WHO strongly recommends Xpert MTB/RIF as the initial diagnostic test in children suspected of having multidrug-resistant or HIV-associated tuberculosis, and only conditionally in all children suspected of having tuberculosis.[9]

Immunodiagnostic tests, such as the tuberculin skin test and interferon-γ release assays—ie, QuantiFERON-TB Gold and T-SPOT.TB—do not depend on the presence of *Mycobacterium tuberculosis* in collected samples. Although these tests have use in screening

special risk populations for latent tuberculosis infection, they cannot enable the crucial distinction between active tuberculosis disease and latent tuberculosis infection.[10] Failure to accurately identify cases with active tuberculosis restricts the clinical application of these tests in endemic regions where latent tuberculosis infection is ubiquitous.

Results of previous studies in adults showed that flow-cytometric analysis of the CD27 on circulating *M tuberculosis*-specific T cells can discriminate active tuberculosis from latent tuberculosis infection.[11–15] Loss of CD27 expression on *M tuberculosis*-specific CD4 T cells is a marker of active tuberculosis due to persistent antigenic stimulation[16] and probably relates to increased cellular homing to the site of disease.[17,18] In a proof-of-concept study, we assessed the performance of the new T-cell activation marker–tuberculosis (TAM-TB) assay for the diagnosis of active tuberculosis in children with symptoms that suggest tuberculosis.

Methods

Study population

This prospective diagnostic assessment was done at two Tanzanian research sites—the NIMR-Mbeya Medical Research Center, Mbeya, and the Ifakara Health Institute, Bagamoyo. Children older than 6 months and younger than 16 years with signs or symptoms that suggested tuberculosis were enrolled from May 10, 2011, until Sept 4, 2012, and followed up for a minimum of 5 months. At least one of the following eligibility criteria had to be met: persistent, non-remitting cough for more than 14 days that did not respond to antibiotics; repeated episodes of fever within the past 14 days that did not respond to antibiotics, after malaria had been excluded; weight loss or failure to thrive during the previous 3 months; and signs and symptoms that suggested extrapulmonary tuberculosis. Children who received tuberculosis treatment in the past 12 months were excluded. The children were referred from peripheral health facilities and local hospitals. Because of cost restrictions, a subgroup of 130 children was selected from a larger cohort of 290 children to ensure testing of at least 20 children with culture-confirmed tuberculosis after prevalence estimation at the two sites. All other clinical information was masked from the investigators doing the selection with a list of patient identification numbers with corresponding disease classifications.

See Online for appendix

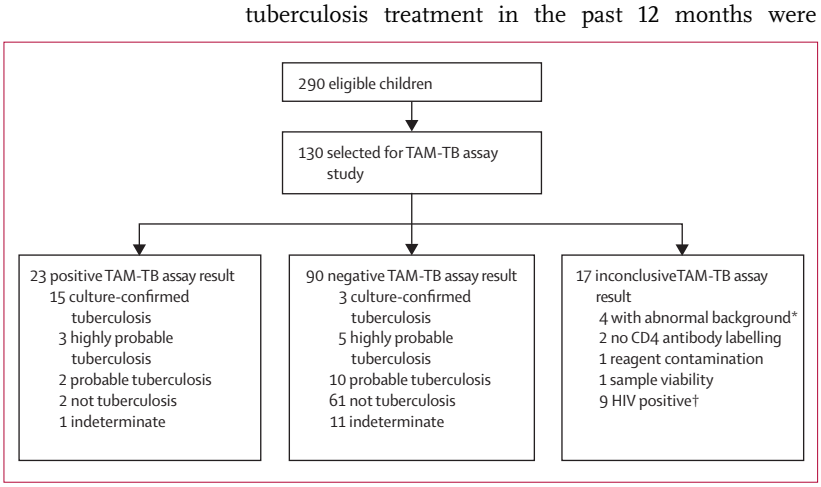

Figure 1: **Study profile**
TAM-TB=T-cell activation marker–tuberculosis. *Frequency of interferon-γ-positive CD4 T cells in the negative control greater than the median frequency plus 3 SD of the tested sample. †CD4 T cell count of less than 10 000 per μL and interferon-γ CD4 T cells less than 0·05%.

The Institutional Review Board of the Ifakara Health Institute, the Mbeya Medical Research and Ethics Committee, and the Medical Research Coordinating Committee of Tanzania approved the study protocol. We obtained written informed consent from a literate parent or legal guardian. In cases of illiteracy, informed oral consent was attested by an independent witness. Children older than 7 years provided assent for participation.

Classification and reference standard

Classification of children was based on the results of the clinical and microbiological assessment as culture-confirmed tuberculosis (culture-positive for *M tuberculosis*), highly probable tuberculosis (chest radiograph consistent with tuberculosis confirmed by two independent reviewers, histology or cytology typical for tuberculosis, or fluorescent or acid-fast bacilli on microscopy), probable tuberculosis (clinically suspected tuberculosis without objective findings as above), not tuberculosis (alternative diagnosis established and clinical resolution without antituberculosis treatment), or indeterminate (any other combination; appendix). Culture-confirmed tuberculosis was used as the reference standard for sensitivity analysis and not tuberculosis as the reference standard for specificity assessment.

Clinical and laboratory procedures

Clinical procedures at enrolment comprised medical history, physical examination, HIV testing, CD4 T-cell count, interferon-γ release assay (QuantiFERON-TB Gold, Cellestis, Melbourne, VIC, Australia), and chest radiography. Chest radiographs were classified as strongly indicating, uncertain, or not tuberculosis by two independent experts from whom all clinical and diagnostic information was masked. Malnutrition was assessed on the basis of weight-for-age *Z* score in children aged up to 10 years (less than –2) and body-mass index in those older than 10 years (<2 SD below normal). If feasible, at least three induced or two expectorated respiratory specimens were obtained on consecutive days. Induced sputum was obtained in accordance with a standard protocol.[19] Fine-needle aspiration biopsies of enlarged lymph nodes were done when clinically indicated, as per standard protocol.[20]

After N-acetyl L-cysteine-sodium hydroxide decontamination, each sputum sample pellet was assessed with microscopy by use of Ziehl-Neelsen staining. At least one sample was inoculated on both liquid (BACTEC

	All patients (n=113)	Culture-confirmed tuberculosis (n=18)	Highly probable tuberculosis (n=8)	Probable tuberculosis (n=12)	Not tuberculosis (n=63)	Indeterminate (n=12)
Age (years)	6·1 (2·1–10·3)	4·6 (1·5–12·7)	8·1 (5·2–11·9)	6 (1·8–10·5)	6·1 (2·0–9·8)	5·4 (2·2–10·5)
Female sex	52 (46%)	8 (44%)	5 (63%)	6 (50%)	27 (43%)	6 (50%)
Symptoms at enrolment						
Cough	108 (96%)	16 (89%)	6 (75%)	12 (100%)	62 (98%)	12 (100%)
Fatigue or lethargy	28 (25%)	8 (44%)	2 (25%)	2 (17%)	16 (25%)	0
Wheezing	16 (14%)	1 (6%)	1 (13%)	3 (25%)	9 (14%)	2 (17%)
Breathing difficulties	47 (42%)	10 (56%)	4 (50%)	8 (67%)	22 (35%)	3 (25%)
Fever	82 (73%)	16 (89%)	6 (75%)	9 (75%)	45 (71%)	6 (50%)
Chest pain	23 (20%)	1 (6%)	0	1 (8%)	18 (29%)	3 (25%)
Haemoptysis	4 (4%)	1 (6%)	0	1 (8%)	1 (2%)	1 (8%)
Enlarged lymph nodes	12 (11%)	5 (28%)	1 (13%)	1 (8%)	5 (8%)	0
Weight loss	55 (49%)	13 (72%)	3 (38%)	5 (42%)	32 (51%)	2 (17%)
Abdominal pains	20 (18%)	5 (28%)	2 (25%)	3 (25%)	9 (14%)	1 (8%)
Malnutrition	60 (53%)	12 (67%)	4 (50%)	5 (42%)	24 (38%)	5 (42%)
HIV infection	33 (29%)	4 (22%)	3 (38%)	7 (58%)*	15 (24%)	4 (33%)
WHO immunological staging						
Not clinically significant	7/33 (21%)	0/4 (0%)	0/3 (0%)	2/7 (29%)	4/15 (27%)	1/4 (25%)
Mild	6/33 (18%)	1/4 (25%)	1/3 (33%)	2/7 (29%)	2/15 (13%)	0/4 (0%)
Advanced	1/33 (3%)	1/4 (25%)	0/3 (0%)	0/7 (0%)	0/15 (0%)	0/4 (0%)
Severe	19/33 (58%)	2/4 (50%)	2/3 (67%)	3/7 (43%)	9/15 (60%)	3/4 (75%)
On antiretroviral therapy at enrolment	13/33 (39%)	2/4 (50%)	1/3 (33%)	2/7 (29%)	7/15 (47%)	1/4(25%)
Positive tuberculin skin test	31/103 (30%)	13/17 (76%)	3/8 (38%)	2/11 (18%)	9/57 (16%)	4/10 (40%)
Positive interferon-γ release assay	27/110 (25%)	13/18 (72%)	1/7 (14%)	3/12 (25%)	8/61 (13%)	4/12 (33%)
Positive tuberculin skin test or interferon-γ release assay	47/112 (42%)	17/18 (94%)	3/8 (38%)	4/12 (33%)	15/63 (24%)	4/11 (36%)

Data are median (IQR), number (%), or n/N (%). *p=0·033 compared with not tuberculosis distribution (Fisher's exact test).

Table 1: **Demographics and clinical characteristics of study participants by classification group**

MGIT 960, Becton Dickinson, Franklin Lakes, NJ, USA) and solid media (Loewenstein-Jensen culture). Positive cultures were confirmed by use of microscopy, and subsequent MPT64 antigen or molecular tests (Genotype MTBC or CM, Hain Lifescience, Nehren, Germany) or both. GenoType MTBDRplus (Hain Lifescience) or phenotypic drug-susceptibility testing (BACTEC MGIT 960 SIRE kit, Becton Dickinson) was used for resistance testing. Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) was done in at least one sputum sample according to the manufacturer's instructions. All tests, including TAM-TB assay, were done by trained laboratory technical staff masked to clinical information and radiological results.

Results from established diagnostic procedures were made available to support clinical management in accordance with national and international guidelines. Results of the experimental TAM-TB assay were not released.

TAM-TB assay

Details of the underlying biological principle of the TAM-TB assay, definition of cutoff values in an optimisation cohort of adults (n=87), and additional background information are provided in the appendix.

Briefly, the TAM-TB assay is used to measure the CD27 phenotype of CD4 T cells producing interferon γ in response to *M tuberculosis* antigens according to a standard intracellular cytokine staining procedure.[14] *M tuberculosis*-specific CD4 T-cell responses were judged positive when at least five CD4 T-cell interferon-γ-producing events were detected, and the proportion of interferon-γ producing CD4 T cells after antigen stimulation was greater than 0·05% and was at least twice the frequency of that in the negative controls.

Well characterised samples from adults (appendix) were used to identify the CD27 median fluorescence intensity (MFI) ratio with optimum discriminatory power between latent tuberculosis infection and active tuberculosis. Cryopreserved peripheral blood mononuclear cells obtained at the baseline visit were stimulated for 12–16 h with a set of overlapping ESAT-6/CFP-10 peptides[21] (Elephants and Peptides, Potsdam, Germany) and purified protein derivative (Statens Serum Institut, Copenhagen, Denmark) before staining with fluorochrome-labelled antibodies. We compared the MFI of CD27 staining on interferon-γ-positive *M tuberculosis*-specific CD4 T cells to the CD27 MFI value for all CD4 T cells to define the CD27 MFI ratio for a sample (appendix). CD27 MFI ratio results were

consistent in 17 independent quality control assessments of two batches of peripheral blood mononuclear cells (appendix).

Adult patients with tuberculosis had significantly higher CD27 MFI ratios than did control patients after ESAT-6/CFP-10 or purified protein derivative stimulation; area-under-operating-characteristic curves were 0·931% and 0·881%, respectively (appendix). We set CD27 MFI ratio thresholds at greater than 5 for ESAT-6/CFP-10 and greater than 13 for purified protein derivative responses to achieve a balanced TAM-TB assay sensitivity of 83·3% (95% CI 68·6–93·0) and specificity of 83·7% (69·3–93·2) in this optimisation cohort. Once these cutoff values were ascertained, samples from paediatric tuberculosis suspects were tested (validation cohort). TAM-TB assays were done at two independent laboratories according to identical standard operating procedures.

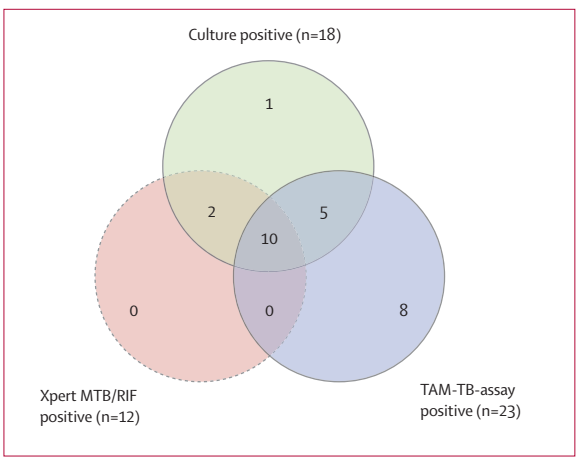

Figure 2: **Venn diagram of positive *Mycobacterium tuberculosis* culture, Xpert MTB/RIF, and TAM-TB-assay results**
TAM-TB=T-cell activation marker–tuberculosis.

	Culture-confirmed tuberculosis (n=18)	Highly probable tuberculosis (n=8)	Probable tuberculosis (n=12)	Not tuberculosis (n=63)	Indeterminate (n=12)
Assay-positive cases	15 (83%)	3 (38%)	2 (17%)	2 (3%)	1 (8%)
Assay-negative cases	3 (17%)	5 (63%)	10 (83%)	61 (97%)	11 (92%)

Table 2: **T-cell activation marker–tuberculosis assay results by classification groups**

	Bagamoyo (n=63)	Mbeya (n=50)	All patients (n=113)
Sensitivity (95% CI)	83·3% (35·9–99·6)	83·3% (51·6–97·9)	83·3% (58·6–96·4)
Positive/total	5/6	10/12	15/18
Specificity (95% CI)	95·6% (84·9–99·5)	100% (81·5–100)	96·8% (89·0–99·6)
Negative/total	43/45	18/18	61/63
Disease prevalence	7/115 (6%)	22/175 (13%)	29/290 (10%)
Positive predictive value	54·9%	100%	74·5%
Negative predictive value	98·9%	97·7%	98·1%

Data are n/N (%), unless otherwise indicated. Culture-confirmed tuberculosis and not tuberculosis were the reference standards for sensitivity and specificity. Calculation of predictive values was based on disease prevalence in the main paediatric cohort (n=290).

Table 3: **Sensitivity, specificity, and predictive values of the T-cell activation marker–tuberculosis assay**

Statistical analysis

Calculation of medians, IQR, test accuracy measures (sensitivity and specificity), Kruskall-Wallis ANOVA with post-test correction (Dunn's), and Mann-Whitney and Fisher's exact tests were done with GraphPad Prism software (version 4.03).

Role of the funding source

The study was funded by the European and Developing Countries Clinical Trials Partnership, which had no role in study design, data gathering, analysis, and interpretation, or writing of the report. The corresponding author had full access to all the data and final responsibility for the decision to submit for publication.

Results

After assay optimisation in adults (optimisation cohort; appendix), the diagnostic performance of the TAM-TB assay was assessed in 130 children suspected to have tuberculosis (validation cohort); samples from 113 (87%) children were eligible for analysis. Figure 1 shows the study profile;[22] 17 (13%) children were excluded from the study because of inconclusive results. 18 (16%) of 113 eligible children had culture-confirmed tuberculosis, eight (7%) highly probable tuberculosis, 12 (11%) probable tuberculosis, 63 (56%) not tuberculosis, and 12 (11%) indeterminate cases (figure 1). A fine-needle aspiration biopsy of enlarged lymph nodes was done in five children; the final classifications were culture confirmed tuberculosis (n=3), probable tuberculosis (n=1), and not tuberculosis (n=1). All culture-confirmed tuberculosis cases were positive on assessment of a respiratory specimen, including one with additional cytomorphology that suggested tuberculous lymphadenitis. Nine (50%) of 18 children with culture-confirmed tuberculosis were sputum smear-positive. Six of eight patients with highly probable tuberculosis had chest radiographs that strongly suggested tuberculosis, one had chronic granulomatous lymphadenitis, and one was sputum smear-positive. Patients with probable tuberculosis had symptoms that suggested infection, which resolved completely on tuberculosis treatment, but radiographic signs and laboratory investigations were non-conclusive.

Table 1 shows the demographic and clinical characteristics of the children. The median age of the 113 children included in the analysis was 6·1 years (table 1). 33 of 113 children had HIV infection (table 1). According to the WHO immunological classification for established HIV infection,[23] 19 of the HIV-infected children had severe immunodeficiency (table 1). 37 of 38 children with culture-confirmed, highly probable, or probable tuberculosis received tuberculosis treatment,

	Clinical case classification and reason for classification	Tuberculin skin test or QuantiFERON	HIV status	First-visit clinical diagnosis, treatment, and response	Follow-up clinical diagnosis, treatment, and outcome
Culture-negative and TAM-TB assay-positive cases					
15 years	Highly probable tuberculosis by radiograph	Positive	Negative	Pneumonia Amoxicillin No	Pulmonary tuberculosis later confirmed by culture and Xpert MTB/RIF 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved
7 years	Highly probable tuberculosis by sputum smear	Positive	Negative	Pneumonia Amoxicillin No	Pulmonary tuberculosis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved
9 years	Highly probable tuberculosis by lymph node cytology	Positive	Positive	Lymphadenitis Ceftriaxone No	Tuberculosis lymphadenitis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved
11 years	Probable tuberculosis by symptoms, resolution after treatment	Negative	Positive	Pneumonia Ceftriaxone No	Pulmonary tuberculosis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved
1 year	Probable tuberculosis by symptoms, resolution after treatment	Positive	Positive	Chest infection Cefalexin No	Pulmonary tuberculosis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved
5 years	Not tuberculosis, no treatment, and healthy after 5 months	Positive	Positive	Pneumocystis pneumonia plus *Mycobacterium interjectum* Co-trimoxazole Resolved	Recovered from initial diagnosis
7 years	Not tuberculosis, no treatment, and healthy after 5 months	Positive	Positive	Pneumonia, urinary tract infection Antibiotics Resolved	Intestinal helminths Albendazole Resolved, new dry cough 6–9 months after recruitment
10 years	Indeterminate	Positive	Positive	Bronchiectasis Metronidazole Moderate	Bronchiectasis, malaria Antimalarial Persisting cough, mediastinal lymphadenopathy
Culture-positive and TAM-TB assay-negative cases					
14 years	Culture-confirmed tuberculosis with *Mycobacterium tuberculosis* in sputum	Positive	Negative	Pulmonary tuberculosis, malnutrition 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Unchanged	Pulmonary tuberculosis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved
8 years	Culture confirmed tuberculosis with *Mycobacterium tuberculosis* in sputum	Positive	Negative	Nephrotic syndrome Furosemide, prednisolone Deterioration, hospital admission	Pulmonary tuberculosis (only retrospectively confirmed) No tuberculosis treatment Death
2 years	Culture confirmed tuberculosis with *Mycobacterium tuberculosis* in sputum	Negative	Negative	Pulmonary tuberculosis, malnutrition 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Unchanged	Pulmonary tuberculosis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved

Positive tuberculin skin test suggested by a lesion of at least 10 mm in HIV-uninfected or at least 5 mm in HIV-infected children. TAM-TB=T-cell activation marker–tuberculosis.

Table 4: **Cases with discrepant culture and TAM-TB assay results: clinical characteristics at enrolment and follow-up visits by age**

and one child died before the positive *M tuberculosis* culture result became available and treatment could be started. 33 of 37 children reported symptom resolution on treatment, one child with highly probable tuberculosis died, and three who were classified as culture confirmed, highly probable, or probable tuberculosis were lost to follow-up. No drug resistance was detected in the culture-confirmed cases.

Respiratory specimens could be collected from each child; 103 (91%) of 113 children provided at least three induced or two expectorated sputum samples. Figure 2 shows the overlap between children with a positive *M tuberculosis* culture, Xpert MTB/RIF, or TAM-TB assay, and table 2 shows the TAM-TB assay results. 15 of 18 children with culture-confirmed tuberculosis had a positive TAM-TB assay result (sensitivity 83·3%, 95% CI 58·6–96·4; table 3). Of the 63 cases classified as not tuberculosis, 61 had a negative TAM-TB assay (specificity 96·8%, 89·0–99·6; table 3). Findings were similar at the two independent sites Bagamoyo and Mbeya (table 3). The sensitivity of the TAM-TB assay was 69·2% (48·2–85·6) when both culture-confirmed and highly probable tuberculosis cases were included in the reference standard.

Table 4 summarises the clinical characteristics, tuberculin skin test and QuantiFERON-TB Gold results, diagnosis at enrolment, response to antibiotic treatment, and clinical follow-up of all children with discrepant TAM-TB assay and *M tuberculosis* culture results. Of the eight children who were TAM-TB assay positive but culture-negative (figure 2), three cases were classified as highly probable tuberculosis, two as probable tuberculosis, two as not tuberculosis, and one as indeterminate; six were HIV infected (table 4). Of the three children with highly probable tuberculosis, one was sputum smear-positive at enrolment, one was culture-confirmed during follow up, and one had cytological signs (chronic granulomatous lymphadenitis) that suggested tuberculosis. In the two probable tuberculosis cases, broad-spectrum antibiotics had no effect, but symptoms resolved on tuberculosis treatment (table 4). The TAM-TB assay was positive in two HIV-infected patients who were classified as not tuberculosis (table 4). *Mycobacterium interjectum* was identified in one case with an induration of 10 mm on the tuberculin skin test and an indeterminate QuantiFERON-TB Gold result. The other patient was QuantiFERON-TB Gold-positive. One HIV-infected child who was classified as indeterminate, had a complex clinical picture with various disease episodes that never fully resolved. Of the three children who were *M tuberculosis* culture-positive, but TAM-TB assay negative, two were malnourished (table 4). The third child had a nephrotic syndrome and died before culture results were available (table 4).

Of the 63 children classified as not tuberculosis, 15 (24%) had a positive tuberculin skin test or QuantiFERON-TB Gold test at enrolment, suggesting probable latent tuberculosis infection. TAM-TB assay was positive in two (13%) of these 15 children. Xpert MTB/RIF enabled detection of 12 (67%) of 18 culture-confirmed tuberculosis cases, giving a sensitivity of 66·7% (95% CI 41·1–85·6) when two or more sputum samples obtained on consecutive days were analysed. The sensitivity was 55·6% (31·3–77·6) when only the first sputum sample was analysed. The specificity of Xpert MTB/RIF was 100% (94·2–100; data were missing for one person). Xpert MTB/RIF was positive in two of three children with culture-confirmed tuberculosis who were TAM-TB assay negative (figure 2). The sensitivity achieved with the combination of TAM-TB assay and Xpert MTB/RIF was 94·4% (72·6–99·1). The median time to detection by use of culture, defined as the period between enrolment and first *M tuberculosis* confirmation, was 19·5 days (IQR 14·0–45·0), whereas TAM-TB assay results were obtained within 24 h after recovery of peripheral blood mononuclear cells.

Discussion

The TAM-TB assay showed good sensitivity and excellent specificity with *M tuberculosis* culture as a reference standard. Specific detection of active tuberculosis in children was based on cutoff values set from the optimisation study in adults. Retrospectively lowering CD27 ratio thresholds to 2 for ESAT-6/CFP-10 and 7 for purified protein derivative stimulation, would further improve assay sensitivity from 83·3% to 88·9% (95% CI 88·0–99·4%) without affecting specificity with additional detection of highly probable (n=1), probable tuberculosis (n=1), and indeterminate (n=1) cases. Hence, cutoff values specifically optimised for children might further improve TAM-TB accuracy. Contrary to molecular-based assays like Xpert MTB/RIF and microbiological tests, the TAM-TB assay can be done on a readily available peripheral blood sample and is not limited by the paucibacillary nature of active tuberculosis in children. Consistent test performance at both study sites suggests the assay is robust and repeatable. Of greatest clinical relevance is that the TAM-TB assay provides an answer within a day of blood collection, which is important because early treatment initiation can be crucial in young children at high risk of disseminated tuberculosis disease.[2,24]

Of the children with discrepant results, five of eight children who were *M tuberculosis* culture-negative and TAM-TB assay positive had highly probable or probable tuberculosis. The clinical classification used might have included some overdiagnosis.[4] International consensus definitions of intrathoracic tuberculosis were not available when the study was designed.[25] However, detailed case assessment suggested that these children probably did have active tuberculosis, suggesting that the TAM-TB assay is probably at least as sensitive as *M tuberculosis* culture.

Despite access to state-of-the-art tuberculosis diagnostics, most children enrolled in our study were

treated for tuberculosis based on a combination of epidemiological and clinical findings. This shows the poor diagnostic usefulness of *M tuberculosis* culture in clinical practice, related to difficult specimen collection, suboptimum sensitivity in children with paucibacillary disease, and long turnaround times. Suboptimum sensitivity undermines the suitability of the test as a reference standard and complicates optimal assessment of diagnostic accuracy. Consistent with previous paediatric studies, Xpert MTB/RIF enabled the detection of culture-confirmed paediatric cases from expectorated or induced sputa with a sensitivity of 66·7% and a specificity of 100%.[26–28] Compared with *M tuberculosis* culture, the combination of Xpert MTB/RIF and TAM-TB, two assays with a turnaround time of less than 24 h, had a sensitivity of 94%, missing one case. This child had severe nephrotic syndrome and culture-confirmation was only achieved after the child had passed away. The combination of TAM-TB assay and Xpert MTB/RIF enabled the detection of more tuberculosis cases than did culture when both culture-confirmed and highly probable tuberculosis cases were included in the reference standard.

The TAM-TB assay was highly accurate in identifying children without tuberculosis, including 13 children with a positive interferon-γ release assay or the result of the tuberculin skin test. Hence, the assay has a high specificity for tuberculosis disease in children despite immunological evidence of previous *M tuberculosis* exposure, consistent with data from the adult optimisation cohort and a previous study in adults.[15] Only two not tuberculosis cases were incorrectly assigned. Both children were infected with HIV, one had simultaneous infection with non-tuberculous mycobacteria and both had latent tuberculosis infection. These false-positive TAM-TB results were caused by a predominance of mycobacteria-specific effector memory T-cell responses in peripheral blood. In view of a positive CFP-10/ESAT-6 response in the child without non-tuberculous mycobacteria infection, the positive TAM-TB assay result might suggest recent *M tuberculosis* infection that did not progress to active disease during the period of observation. In the other case, the non-tuberculous mycobacteria (*M interjectum*) identified in the sputum might have elicited the positive TAM-TB assay result. Implementation of additional, highly immunogenic *M tuberculosis*-specific antigens in the next generation TAM-TB assay could further improve specificity and sensitivity.

The current version of the TAM-TB assay has several limitations, mainly related to cost and technical complexity. It needs advanced blood processing procedures, antigenic stimulations, flow cytometry equipment, and well trained staff. Refinement and simplification are in progress to optimise diagnostic performance and make it compatible with cytometers that are in widespread use for measurement of CD4 T-cell counts in HIV/AIDS-affected countries. The TAM-TB assay did not generate valid test results in 13% of the samples of peripheral blood mononuclear cells, but this might be attributed to reduced T-cell viability and decreased cytokine production after cryopreservation.[29] Ideally, the TAM-TB assay should be done on fresh whole blood samples—eg, in antigen-precoated tubes similar to the commercial QuantiFERON In-Tube system (Cellestis).[30] Low CD4 T-cell counts due to severe HIV infection might remain a problem even when fresh blood samples are used. The small sample size of this proof-of-concept study resulted in very wide confidence intervals and did not allow systematic assessment of test performance in very young, malnourished, or HIV-infected children who have recently been started on antiretroviral therapy. These factors might negatively affect the accuracy of the TAM-TB assay.[15,31] Additional studies need to be done to specifically address test performance in these patient groups. The assessment of the TAM-TB assay in a study population enriched for culture-confirmed tuberculosis cases might have been a source of bias, particularly for the analysis including the under-represented classification groups. Although the TAM-TB assay cannot provide information about drug susceptibility, use of concomitant Xpert MTB/RIF testing can address this limitation.

***Panel:* Research in context**

Systematic review

We searched PubMed up to June 10, 2014, using the terms "CD27", "diagnosis", "human", and "tuberculosis". Of the 33 results returned, seven were clinical studies from five independent laboratories and their results showed the potential of monitoring CD27 expression on *Mycobacterium tuberculosis*-specific CD4 T cells to diagnose active tuberculosis disease in adults. We did not find a report of the assessment of the use of *M tuberculosis*-specific T-cell phenotype for the diagnosis of active childhood tuberculosis.

Interpretation

Our report is the first to assess the accuracy of using CD27-expression analysis for the diagnosis of paediatric tuberculosis. Immunological data were generated and analysed by investigators from whom clinical information was masked. The T-cell activation marker–tuberculosis assay showed excellent sensitivity (83·3%) and specificity (96·8%) when *M tuberculosis* culture positivity was used as a reference standard, and enabled the detection of additional cases in culture-negative children who were clinically suspected of having tuberculosis. Despite a fairly small sample size and potential bias originating from cohort enrichment in culture-confirmed tuberculosis cases, the results of this proof-of-concept study show that immunodiagnostic tests that incorporate phenotypic characteristics of *M tuberculosis*-specific T cells have the potential to improve rapid detection of active tuberculosis in children.

To our knowledge, this study is the first to assess the diagnostic performance of the novel TAM-TB assay in children (panel). Importantly, it was done in a region with a high tuberculosis incidence and recruited children with symptoms that suggested tuberculosis who represent the real-life diagnostic challenge in these settings. Despite a need for further refinement and testing in other regions with high burden of tuberculosis, our results suggest that the sputum-independent TAM-TB assay is a major advance for the rapid and accurate diagnosis of tuberculosis in children.

Contributors

KR, MH, and CG designed the study. CG designed the TAM-TB assay. PC, ES, AR, NEN, EM, KS, FH, FL, LJ, MH, and KR oversaw enrolment, patient care, or standard laboratory work. DP, FM, AB, MC, MM, CG, and CD were responsible for the flow-cytometry analysis. DP, FM, ES, KR, and CG did the data management and analysis. DP, KR, CG, CD, and BJM wrote the draft of the report. BJM provided expert advice. All authors contributed to data gathering and interpretation, and revision of the report.

Declaration of interests

We declare no competing interests.

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