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Stool Xpert MTB/RIF test for the diagnosis of childhood pulmonary tuberculosis at primary clinics in Zimbabwe

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Running head – Xpert MTB/RIF testing on stool from children

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Abstract

**Objective**- To evaluate the diagnostic performance of Xpert MTB/RIF on stool samples from children clinically suspected of pulmonary tuberculosis at primary care clinics.

**Design**- A cross-sectional diagnostic study enrolling 5-16 year olds from whom one induced sputum sample was tested for microbiological tuberculosis confirmation. Results of a single stool sample tested on Xpert MTB/RIF were compared against microbiologically confirmed tuberculosis defined as a positive result on smear microscopy and/or culture and/or induced sputum Xpert MTB/RIF.

**Results**- Of the 222 children enrolled, 218 had complete microbiological results. Median age was 10.6 (IQR: 8-13) years. Tuberculosis was microbiologically confirmed in 19 (8.7%) of the 218 children. Of these, 5 (26%), 9 (47%) and 15 (79%) were smear, culture and induced sputum Xpert MTB/RIF positive, respectively. Stool Xpert MTB/RIF testing detected 13 (68%) of the 19 microbiologically confirmed cases and was negative in 195 (98%) of 199 microbiologically negative cases. Stool Xpert MTB/RIF detected more of HIV-infected than in HIV-uninfected children, 76.9% (10/13) versus 50% (3/6).

**Conclusion**– Stool Xpert MTB/RIF could be a potential alternative screening test for children suspected to have tuberculosis if sputum is unavailable, particularly in HIV-infected children. Strategies to optimise diagnostic yield of the stool Xpert MTB/RIF assay need further study.

**Key words** - Genexpert, faecal specimen, children
**Introduction**

Childhood tuberculosis (TB) contributes about 10% of the total 9 million incident cases worldwide, with an estimated 136,000 deaths in children in 2014 \(^1\). The burden of TB in children is largely due to undiagnosed and late diagnosis of adult TB which creates a reservoir for transmission to children \(^2\).

The major challenge to timely diagnosis of TB in children is the difficulty in obtaining spontaneously expectorated sputum, necessitating the use of sputum induction, which requires skill and infrastructure. Further, sputum from children is often paucibacillary as children are less likely to form cavitary lesions in lungs to contain the TB bacilli \(^2,3\). Consequently, the sensitivity of standard diagnostic tests, sputum microscopy and culture, is lower. A more easily accessible sample that can be obtained with minimal skill at primary health care level and an alternative diagnostic tool is therefore required for the diagnosis of TB in children.

*Mycobacterium tuberculosis* deoxyribonucleic acid (DNA) can be detected by an automated molecular assay, Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA). The assay is automated and includes sample processing, nucleic acid extraction, polymerase chain reaction and detection of specific codon sequences of *Mycobacteria tuberculosis* within a closed system \(^4,5\). The assay has the advantage of producing a result in 3 hours with little hands on time in sample processing.

The Xpert MTB/RIF assay has shown high sensitivity in previous studies when sputum \(^6,7,8\), gastric lavage \(^9,10\), broncho-alveolar lavage \(^11\) and nasopharyngeal aspirates \(^12\) were used. As a result the assay has been endorsed by the World Health Organisation as an initial test for diagnosing TB in children. However, invasive procedures are required to obtain such samples.

Stool is a potential alternative sample that can be collected non-invasively. Children tend to swallow sputum when they cough and *Mycobacterium tuberculosis* DNA has been shown to survive the harsh acidic and digestive environment of the gastro-intestinal tract \(^13\). We therefore evaluated the diagnostic performance accuracy of Xpert MTB/RIF using stool in children with clinically suspected pulmonary TB presenting to primary care clinics in Harare, Zimbabwe. The study aimed to determine the sensitivity and specificity of Xpert MTB/RIF testing on stool against confirmed microbiological pulmonary TB determined by testing induced sputum (IS) with microscopy, solid culture and Xpert MTB/RIF.

**Methods**
Participants

Participants were recruited from eight primary care clinics in Harare between September 2013 and October 2014, with guardian consent and participant assent. These were children aged 5 to 16 years presenting with a chronic cough of more than 2 weeks and any one of the classical signs and symptoms of TB, including weight loss, loss of appetite, persistent fever without an apparent cause, night sweats or history of close contact with a TB index patient, defined as living in close proximity (sharing a room within a household) with an adult diagnosed with tuberculosis within the preceding twelve months. Those aged 0 to 5 years were not included as there was insufficient evidence on safety of sputum induction in this age-group, at the time of seeking study approvals, to satisfy the Harare City Health approval committee. Evidence had been especially requested for this vulnerable age-group as sputum induction is not commonly practised in Zimbabwe. Participants were excluded if they had an already confirmed TB diagnosis, were on anti-TB treatment for more than 72 hours prior to enrolment, required emergency medical attention or were resident outside Harare.

Study procedures

A structured questionnaire was used to collect data on demographics and physical examination. HIV status was obtained from documented proof of test result. In case of unknown HIV status, participants were offered the test through the clinic’s HIV testing protocols. All participants were asked to provide a stool sample and to undergo sputum induction to obtain one sputum sample.

Sputum induction was performed by a trained nurse following the method described by Zar et al., (14) except where it was contraindicated (15). The procedure was performed in an open area after a 2-3 hour fast. Two doses of 100µg salbutamol were administered via an aero-chamber to prevent bronchoconstriction, followed by nebulisation with 5% hypertonic saline using a portable nebulisation unit, for five minutes or until the participant started to cough. Oxygen saturation was monitored during and 30 minutes after the procedure. Induced sputum (IS) was collected into a sterile container, using nasopharyngeal suctioning with a sterile mucus extractor if required. If the participant failed to produce sputum the procedure was repeated once, five minutes after the first attempt.
The stool sample (about 5g) was collected into a wide mouthed specimen collection jar on spot or submitted the following day. Samples were transported at 4-8°C to the laboratory within 8 hours of collection.

Anti-TB treatment was commenced by the residing medical officer at the clinic following national TB guidelines, where diagnosis is based on clinical history and examination and/or chest radiography and/or microbiological results. Study test results on microbiological confirmation were made available to the medical officer to assist them in participant management.

Laboratory methods

The sputum sample was processed for microscopy, culture and Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) testing within 24 hours of collection. It was decontaminated by adding an equal volume of 4% sodium hydroxide for 15 minutes and neutralised with phosphate buffered saline (PBS). The mixture was centrifuged at 3200g for 20 minutes to obtain a sputum sediment which was re-suspended in 1.5ml PBS. A drop was used to make smears stained initially using Auramine O method for fluorescent microscopy and confirmed by ZN staining. All smears were re-read by a second reader blinded to the first reading. Discordant results were resolved through a third reading by a different reader. A 0.1ml of IS sediment was cultured on Lowenstein Jensen slopes at 37°C for eight weeks with weekly monitoring for growth. At least one colony on Lowenstein Jensen slope was considered to be positive *Mycobacterium tuberculosis* growth, confirmed by ZN stain and speciation using the SD Bioline MPB64 antigen rapid test. Discordant ZN positive/MPB64 antigen negative samples were speciated using colony morphology, growth at different temperatures and growth on paranitrobenzoic acid containing Lowenstein Jensen slopes. Confirmed cultures underwent phenotypic direct susceptibility testing on Lowenstein Jensen slopes for 4weeks at 37°C. For Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) testing, 0.5ml of IS sediment was mixed 1.5ml Xpert MTB/RIF reagent and mixture tested according to manufacturer’s instructions.

Stool samples were processed within two days of collection as described by Nicol *et al.* (16). Briefly, an aliquot of 0.15g was measured into a screw capped centrifuge tube using a sterile disposable plastic loop. The loop was left inside the tube, 2.4ml of PBS added and the mixture vortexted to remove stool particles from the loop and emulsify the sample. The loop was removed and sample mixture left undisturbed at room temperature for 20 minutes after which
two aliquots of 1ml supernatant were removed. One aliquot was used for immediate testing and another stored for repeat testing if required. Prior to testing, the processed stool sample was centrifuged at 3200g for 15 minutes, sediment re-suspended in 1ml PBS, mixed with 2ml Xpert MTB/RIF reagent and tested according to the manufacturer’s (Cepheid, Sunnyvale, CA, USA) instructions. Interpretation of results was auto-generated by the Xpert MTB/RIF system, and personnel carrying out the index test were blinded to sputum smear and culture test results.

Data analysis
The sample size was based on an anticipated sensitivity of the stool MTB/RIF test of 80%, level of precision set at 10% and an anticipated loss to follow up of 4%. At the time of computation there was no data on prevalence of TB in children in our setting, therefore prevalence was set at 29% adopted from a community study of paediatric TB contacts in South Africa(17).

Data was entered into EpiData version 3.1 (Odense, Denmark) and analysed using STATA version 13.0 (StataCorp, Texas, USA). Descriptive statistics were used to characterise the study population. The Chi square test was used to test for associations. The performance of stool Xpert MTB/RIF was assessed as the proportions of stool Xpert MTB/RIF positive cases amongst microbiologically defined TB status and clinically diagnosed TB cases. Microbiological TB was defined as a positive result on either ZN smear microscopy and/or culture and/or Xpert MTB/RIF test on IS. Indeterminate TB status results and samples without complete data on index and reference tests were excluded from analysis. The study proposed to evaluate the performance of stool Xpert MTB/RIF in TB clinical case definitions as defined by Graham et al., 2012 (18). However, the number of children reporting having a chest X-ray done was minimal (n = 24) to provide meaningful classification.

Ethical approval for the study was obtained from the Harare City Health Department, Joint Parirenyatwa Hospital and College of Health Sciences Research Ethics Committee and by the Medical Research Council of Zimbabwe.

Results
A total of 222 participants were recruited. Four participants were excluded from the analysis: one participant was withdrawn by the guardian, two participants were unable to provide
induced sputum (IS) samples and one participant had an IS Xpert MTB/RIF test that produced an error result despite repeated testing.

The median age of the 218 remaining participants was 10.6 (IQR: 8 -13) years and 44% were male. The median z-scores for weight-for-age and height-for-age were -1.09 (IQR: -1.99 to -0.46) and -1.88 (IQR: -3.70 to -0.73) respectively. HIV status was available for 201 (91%) participants, of whom 111 (51%) were HIV infected, with 55 (49.5%) taking antiretroviral therapy (ART). The distribution of demographic characteristics between microbiological TB and no TB groups is shown in Table 1.

Microbiologically confirmed tuberculosis

The overall prevalence of TB by microbiological confirmation, defined as a positive result on either ZN smear microscopy and/or culture and/or Xpert MTB/RIF test on IS, was 8.7% (19/218). Five (26%) out of the 19 microbiologically confirmed TB cases were less than 10 years old. Of the 19 microbiologically confirmed TB cases, 5 (26%) were smear positive, 9 (47%) were culture positive and 15 (79%) were Xpert MTB/RIF positive on IS. Only two (10.5%) out of 19 microbiologically confirmed TB cases were positive by all 3 microbiological tests (Figure 1). Amongst the culture positive children, only 2 (22%) were smear positive. Rifampicin resistance was not detected in any of the TB cases either by direct susceptibility testing or Xpert MTB/RIF testing. A positive HIV status was documented in 13 (68%) of the 19 microbiological TB cases. Of these 13 children, 7 (54%) children were on ART and 11 (85%) children tested positive with Xpert MTB/RIF sputum (Figure 1).

A total of 32 (14.7%) out of 218 children were commenced on anti-tuberculosis treatment, 19 (59.4%) of whom were microbiologically confirmed and 13 (40.6%) clinically diagnosed by the attending clinician.

Diagnostic performance of the stool Xpert MTB RIF

Xpert MTB/RIF on stool was positive in 17 (8.3%) of 218 children. Of these, 13 (76.5%) had microbiologically confirmed TB. Xpert MTB/RIF stool detected 60%, 66.7% and 86.7% of smear positive, culture positive and IS Xpert MTB/RIF positive cases of TB respectively. Of the 13 microbiologically confirmed TB cases with HIV, Xpert MTB/RIF on stool identified 10 (76.9%) cases. Stool Xpert MTB/RIF detected 13 (68%) of the 19 microbiological confirmed
TB cases and was negative in 195 (98%) of the 199 children without microbiological confirmation (Table 2). Notably, stool Xpert MTB/RIF performed better in children aged 10 years and older compared to those less than 10 years, detecting 71.4% (10/14) and 60% (3/5), respectively. Thirteen children with a clinical diagnosis and commenced on anti-tuberculosis treatment included two (50%) of the four children with a positive stool Xpert MTB/RIF test and without microbiological confirmation. These two children were both HIV-infected and older than 10 years (Table 2).

**Discussion**

The main finding of this study is that Xpert MTB/RIF testing on stool samples identified 68% of microbiologically confirmed TB and was negative in 98% of children with negative
microbiologically results. In addition, similar to findings by Nicol et al., (16) this study showed that Xpert MTB/RIF on stool was more sensitive in HIV-infected than in HIV-uninfected children. Although the test failed to identify 32% of TB cases, it may be of more value in HIV-infected children where there is a high risk of acquiring TB, as well as high risk of disease progression. The routinely available test at primary care centres is smear microscopy which has a sensitivity of less than 10-15% in children with proven TB (19-21) and performs even more poorly in those with HIV infection (22).

The Xpert MTB/RIF is currently being rolled out in many parts of southern Africa for use as an initial test for the diagnosis of TB. The test detects 2 to 3 times more cases than smear microscopy in children (7-9;20) and additionally provides rapid diagnosis in less than 3 hours. However, its limitation, as well as that of microscopy, is that it requires a sputum sample which is difficult to obtain freely expectorated from children. At primary care level induction of sputum from children is rarely attempted and when performed the quality of specimen varies with personnel which results in variable test sensitivity. Findings by Welday et al., 2014 showed that testing of stool samples from children using Xpert MTB/RIF test detected all of the sputum smear positive cases (23). In this study, we have shown that stool Xpert MTB/RIF test identified most of the IS Xpert MTB/RIF positive cases suggesting that when a sputum sample is not readily available a stool sample which is easier to collect may be used with Xpert MTB/RIF test. However, the diagnostic yield of stool Xpert MTB/RIF test still requires optimisation.

The inability of stool Xpert MTB/RIF to detect six of the children with microbiological confirmation may possibly be due to the presence of polymerase chain reaction inhibitors present in stool samples which may have interfered with detection of TB in cases. Of late, there is emerging evidence that the use of larger sample volumes of 2cm³ (24) and 0.6g (25) and pretreatment with a stool processing buffer to inactivate polymerase chain reaction inhibitors (25) achieves greater diagnostic yield of stool Xpert MTB/RIF test.

Stool Xpert MTB/RIF test performed poorly in the clinically diagnosed children. This finding concurs with that of Detjen et al., 2015 in a review of Xpert MTB/RIF testing in children using sputum and gastric aspirates, where the assay showed poor performance in clinically defined probable TB cases (26). This potentially indicates a limitation of the assays’ detection limit rather than of the sample used as clinical microbiologically negative TB cases are more likely to have paucibacillary disease. The two clinically diagnosed children identified by stool Xpert
MTB/RIF were both HIV-infected, again indicating the potential use of the test in HIV infection.

The limitation of our study was the lower than expected TB prevalence, which limits study power. Secondly, we used one stool sample collected at random. The use of a second sample has been shown to have incremental value in detecting additional cases (6;7;21). Care should be taken when interpreting our findings given that the performance of stool Xpert MTB/RIF was assessed in microbiologically confirmed TB and in children 5 to 16 years old. Different results may be obtained for clinical cases and children less than 5 years in whom TB disease presentation is markedly different.

In conclusion, the stool Xpert MTB/RIF holds promise as an alternative test to Xpert MTB/RIF sputum testing, particularly in HIV-infected children. Stool collection is easier and relatively safe compared to sputum collection and may potentially have diagnostic utility if sensitivity is optimised. Further studies are required to determine optimal stool collection times, optimal sample volumes, the need for a second sample, concentration techniques that allow maximum yield and test combination algorithms.

Acknowledgements

The authors are grateful to study participants, their guardians and the research team. The study was funded by the Welcome Trust through the Southern Africa Consortium for Research Excellence (SACORE) initiative.

M Chipinduro developed the study concept and proposal, supervised the study and was responsible for the main writing of the paper. K Mateveke supervised the data analysis. B Makamure supervised the microbiological tests. RA Ferrand assisted with proposal writing and revised the manuscript writing critically for intellectual content. E Gomo was the academic supervisors for M Chipinduro and supervised the whole research process from proposal writing to manuscript writing. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.
Reference List


Tables
### Table 1: Baseline characteristics for TB and non TB patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>TB patients (n = 19)</th>
<th>No TB patients (n = 199)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (IQR)</td>
<td>13 (9 -15)</td>
<td>11 (8 -13)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>12 (63)</td>
<td>111 (56)</td>
<td>0.56</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-&lt;10</td>
<td>5 (26)</td>
<td>95 (48)</td>
<td>0.07</td>
</tr>
<tr>
<td>≥10-16</td>
<td>14 (74)</td>
<td>104 (52)</td>
<td>0.07</td>
</tr>
<tr>
<td>History of contact</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4 (21)</td>
<td>47 (24)</td>
<td>0.77</td>
</tr>
<tr>
<td>History of TB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2 (11)</td>
<td>15 (8)</td>
<td>0.65</td>
</tr>
<tr>
<td>HIV status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>13 (68)</td>
<td>a98 (55)</td>
<td>0.28</td>
</tr>
<tr>
<td>On ART</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7 (54)</td>
<td>48 (49)</td>
<td>0.74</td>
</tr>
<tr>
<td>Presenting symptoms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fever</td>
<td>14 (74)</td>
<td>b91 (47)</td>
<td>0.03</td>
</tr>
<tr>
<td>weight loss</td>
<td>c13 (81)</td>
<td>d109 (60)</td>
<td>0.10</td>
</tr>
<tr>
<td>night sweats</td>
<td>e8 (44)</td>
<td>f76 (40)</td>
<td>0.74</td>
</tr>
</tbody>
</table>

This table includes 218 children (19 microbiologically confirmed TB patients and 199 non TB patients) and excludes 4 children without microbiological results.

a Excluded 20 no TB patients without documented proof of HIV test, b Excluded 5 no TB patients who did not know history of fever, c excluded 3 and d18 TB and no TB patients respectively who did not know history of weight loss, excluded e1 and f11 TB and no TB patients respectively who did not know history of night sweats.

TB is Tuberculosis (pulmonary), HIV is human immunodeficiency virus and ART is anti-retroviral therapy.
Table 2: Diagnostic performance of stool Xpert MTB/RIF in microbiologically determined TB status and in clinically diagnosed TB cases

<table>
<thead>
<tr>
<th>Microbiological TB Result N = 218</th>
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<tr>
<td></td>
<td>All</td>
<td>HIV+</td>
<td>HIV-</td>
<td>&lt;10yr</td>
</tr>
<tr>
<td></td>
<td>N=19</td>
<td>*N=9</td>
<td>*N=8</td>
<td>s</td>
</tr>
<tr>
<td>Positive</td>
<td>n(%</td>
<td>n(%)</td>
<td>n(%)</td>
<td>N=5</td>
</tr>
<tr>
<td>HIV+</td>
<td>13</td>
<td>10</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>HIV-</td>
<td>(68)</td>
<td>(76.9)</td>
<td>(50)</td>
<td>(60)</td>
</tr>
<tr>
<td>≥10yr</td>
<td>32.6</td>
<td>(23.1)</td>
<td>(50)</td>
<td>(40)</td>
</tr>
<tr>
<td>Stool Xpert MTB/RI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Test</td>
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</table>

<table>
<thead>
<tr>
<th>Clinically Diagnosed TB</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>HIV+</td>
<td>HIV-</td>
<td>&lt;10yr</td>
</tr>
<tr>
<td></td>
<td>N=13</td>
<td>*N=7</td>
<td>*N=6</td>
<td>s</td>
</tr>
<tr>
<td>HIV+</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HIV-</td>
<td>(15.4)</td>
<td>(28.6)</td>
<td>(0.0)</td>
<td>(0.0)</td>
</tr>
<tr>
<td>≥10yr</td>
<td>(84.6)</td>
<td>(71.4)</td>
<td>(100)</td>
<td>(100)</td>
</tr>
<tr>
<td>Stool Xpert MTB/RI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
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<tr>
<td>Test</td>
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</tbody>
</table>

TB is tuberculosis (pulmonary), HIV is human immunodeficiency virus, + is positive, - is negative and n is the number of correct results expressed as a percentage (%).

* 20 children without HIV test result excluded; ^ 3 children without HIV test result excluded. Two of the 4 children with a negative microbiological test result and stool Xpert MTB/RIF positive received a clinical diagnosis.
Figure Legend

Figure 1: Venn diagram for positive smear microscopy, culture and Xpert MTB/RIF on induced sputum (IS). This diagram includes 19 children with microbiological tuberculosis. Numbers in brackets indicate HIV infected cases. All the 19 microbiological confirmed children received anti-tuberculosis treatment.
Figure 1

Smear microscopy

Culture

Xpert MTB/RIF on IS

Smear microscopy

Culture

Xpert MTB/RIF on IS