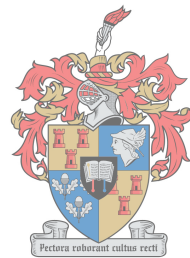


**INNOVATIVE STRATEGIES TO IMPROVE THE DIAGNOSIS OF INTRATHORACIC  
TUBERCULOSIS IN CHILDREN**

By

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## Summary

Paediatric tuberculosis (TB) contributes approximately 10% of the global TB burden, with over one million estimated new cases and 253,000 TB-related deaths in children during 2016. Paediatric TB is a particular problem in low and middle income countries. However, the majority of paediatric cases were not notified to National TB Programs or the World Health Organization and >96% of deaths were estimated to have occurred in children who were not receiving antituberculosis treatment. Young, HIV-infected and malnourished children progress rapidly from infection with *Mycobacterium tuberculosis (M.tb)* to TB, and are at exquisite risk of significant morbidity and mortality from complicated and disseminated forms of TB. The challenges around the diagnosis and microbiological confirmation of pulmonary TB (PTB), the most common manifestation of TB disease in children, contribute to poor access to appropriate treatment and to under-reporting.

The diagnosis of TB in young children typically relies on the evaluation of clinical symptoms and epidemiological factors, and, if available, includes tests of TB infection and chest radiology. All of these tools have considerable limitations and cannot reliably confirm or exclude a diagnosis of PTB. However, the bacteriological confirmation of PTB in children requires the collection of respiratory specimens using procedures that are both relatively invasive and resource-intensive. Furthermore, the current gold standard of diagnosis, mycobacterial culture, has low sensitivity (approximately 30%) and long turnaround time (up to 6 weeks) in children, who typically have paucibacillary TB (low bacillary load). In resource-limited settings, the capacity for respiratory sampling of young children is typically low. These diagnostic challenges prevent adequate reporting and global surveillance of paediatric TB. Diagnostic uncertainty also compromises the

clinical management of paediatric PTB, resulting in over- and under-treatment, and has resulted in the systematic exclusion of children from much-needed interventional research, including tuberculosis treatment trials. Diagnostic research in paediatric PTB has also been poorly standardised, making generalizability and comparability of results difficult. In addition, the insensitive reference standard has hindered progress towards the development of new diagnostic tests tailored for children.

In an effort to develop and investigate more feasible strategies to improve and promote microbiological testing of children with suspected PTB living in high TB-burden settings, I enrolled a large well-characterized cohort of children presenting to hospital with suspected PTB. Children were thoroughly investigated, using standard approaches and intensive specimen collection for liquid culture and molecular testing by Xpert® MTB/RIF (Xpert). Chest radiographs were dual read by blinded experts and reported using standard forms. All children were followed regardless of their final diagnosis and the spectrum of TB and non-TB disease was well described. I evaluated a number of novel diagnostic strategies, including the use of stool specimens for diagnosis of PTB using culture and Xpert, using different stool processing methods, and pooling respiratory specimens to improve the diagnostic yield and reduce the cost of laboratory testing. Importantly, I developed a framework for future evaluation of novel diagnostic tools/biomarkers for the diagnosis of PTB in children.

The total cohort included 608 children and was representative of the demographics and spectrum of disease observed in many high TB-burden settings, where young children bear the highest burden of TB disease. The median age of the cohort was 16.2 months, with 11.8% HIV-infected. Infants below 6 months of age constituted almost 15% of the total cohort. More than 20% of children had a non-specific clinical presentation, with similar prevalence of acute respiratory symptoms across all age

groups and diagnostic categories. Radiological features not typically associated with PTB were common, and indicate a high burden of respiratory pathology as well as potentially non-typical radiological manifestations of PTB. Two hundred and eighty-one (46.2%) children were diagnosed with PTB and were prescribed antituberculosis treatment: 117 (41.6%) were microbiologically confirmed by Xpert or culture, which represents a high diagnostic yield, considering that approximately 50% of children with PTB had non-severe pulmonary disease. In addition, 20/327 (6.6%) children initially considered symptomatic controls were initiated on antituberculosis treatment within two months of enrolment, due to poor clinical progress or positive results from baseline and follow-up bacteriological investigations. This emphasizes the importance and utility of careful specimen collection, incorporating different specimen types and different diagnostic tests and of follow-up of all children in whom there is a clinical suspicion of PTB.

An unexpectedly high proportion of young infants <6 months of age had severe PTB, including cavities, associated with high bacillary load and smear-positivity. In addition, young infants and HIV-infected children were high-risk groups for disseminated TB. This calls for urgent priority to be given towards the development of tailored diagnostic tests that can rapidly confirm and quantify *M.tb* disease in the youngest children, and in the early stages of disease, prior to rapid progression to severe TB. Careful consideration should be given to infection control measures when managing and investigating children, including young infants with suspected PTB.

I showed that stool as a specimen was useful to confirm *M.tb* using Xpert in children with severe pulmonary disease, particularly in children with cavities on chest radiograph, detecting 45% of those who were bacteriologically confirmed on respiratory specimens. A novel centrifugation-free processing method for stool specimens (stool processing kit) showed similar results to the more laborious, centrifugation-dependent

methods I initially investigated. This new approach could be used with more sensitive molecular assays in future to improve stool-based diagnosis of PTB in children. In contrast, stool culture had limited value in the detection of *M.tb*, primarily due to very high contamination (>41% of stool cultures) using standard N-acetyl-l-cysteine-sodium hydroxide (NALC-NaOH 1.25%) decontamination protocols.

Finally, I showed that pooling up to three respiratory specimens of different types (gastric aspirate, induced sputum and nasopharyngeal aspirate) per child, in children who could not expectorate sputum, had similar diagnostic yield by Xpert and culture as individually testing the same three single respiratory specimens. In paired analyses, pooled specimens had significantly higher overall yield than induced sputum and nasopharyngeal aspirate alone, but had similar diagnostic yield as a single gastric aspirate (86.5% vs. 74.4% respectively,  $p=0.46$ ). The overall yield of three individual specimens tested individually was 86% of all confirmed cases, similar to the overall yield of pooled specimens. These results support the substantial diagnostic value of a single gastric aspirate using culture and Xpert, and of “front-loading” specimens of different types on one day to improve the feasibility of specimen collection in young children.

Through this cohort study, I collected comprehensive follow-up data documenting response to antituberculosis treatment and clinical progress in children not receiving antituberculosis treatment (symptomatic controls), to 6 months. These data will be further analysed to validate recently proposed clinical case definitions for TB diagnostic research in children, including the diagnostic value of clinical and other follow-up measures. Symptomatic controls who initially presented with symptoms suggestive of PTB will be further analysed to better understand the spectrum of non-TB respiratory disease borne by children from high-TB burden settings. I have also established a bio-

repository of well-characterised blood and urine specimens for evaluation of promising diagnostic and prognostic biomarkers of TB disease in children.

In summary, through this body of research, I have generated novel data on the utility of several feasible diagnostic strategies for the diagnosis of PTB in HIV-infected and uninfected children from high TB-burden settings. I have analysed these data in relation to relevant clinical and laboratory characteristics in order to make specific recommendations on the most appropriate placement of these strategies, considering both target populations and different levels of health care. I was able to do this by carrying out a well-designed study, in a well-described cohort and by comprehensively reporting on all aspects of the study, including non-evaluable results and complex clinical scenarios. These aspects should be considered when future diagnostic studies for paediatric PTB are being designed, implemented and reported. I have created a rigorous framework for the evaluation of future novel diagnostic strategies, and I have identified numerous areas which require further research and intervention.

## Opsomming

Tuberkulose (TB) in kinders dra by tot ongeveer 10% van die globale las van TB ter wêreld, met ongeveer 1 miljoen nuwe gevalle en 'n geskatte 253,000 TB-verwante sterftes in kinders in 2016. Tuberkulose in kinders is hoofsaaklik 'n probleem in middle- en lae-inkomste lande. Die meerderheid van kindertuberkulose gevalle word egter nie aangemeld aan Nasionale Tuberkulose Kontroleprogramme, of die Wêreld Gesondheid Organisie (WGO) nie, en meer as 96% van die geskatte TB sterftes onder kinders was in kinders wat nie antituberkulose behandeling ontvang het nie. In jong kinders, MIV-geïnfekteerde en wangevoede kinders gaan TB vinning oor vanaf infeksie met *Mycobacterium tuberculosis* (M.tb) na TB siekte, en hierdie kinders het 'n besondere hoë risiko vir betekenisvolle morbiditeit en mortaliteit as gevolg van gekompliseerde en gedissemineerde vorms van TB. Die uitdagings rondom die diagnose en mikrobiologiese bevestiging van pulmonale TB (PTB), die mees algemene vorm van TB in kinders, dra verder by tot die wêreldwye swak toegang tot toepaslike behandeling en die onderberigging van tuberkulose in kinders.

Die diagnose van PTB in jong kinders maak tipies staat op die evaluering van kliniese simptome en epidemiologiese faktore, en, indien beskikbaar, sluit dit ook toetse van TB infeksie en borskasplate in. Al hierdie hulpmiddels het aansienlike beperkinge en kan nie betroubaar die diagnose van TB bevestig of uitsluit nie. Die bakteriologiese bevestiging van PTB in kinders vereis egter die versameling van respiratoriese monsters, wat beide relatief indringend en hulpbron-intensief is. Verder het die huidige goudstandaard van TB diagnose, naamlik kultuur van *Mycobacterium tuberculosis*, 'n lae sensitiwiteit (ongeveer 30%) en het 'n lang omkeertyd (tot 6 weke) in kinders wat TB kry, met 'n lae hoeveelheid *Mycobacterium* organismes. In omgewings met min



hulpbronne, is die kapasiteit om respiratoriese monsters in jong kinders te versamel, tipies laag. Hierdie diagnostiese uitdagings voorkom dat daar toepaslike verslaggewing en globale waarneming is oor kindertuberkulose is. Hierdie diagnostiese onsekerheid ondermyn ook die kliniese sorg van PTB in kinders, wat tot oor-en onderbehandeling lei. Tot dusver het dit ook gelei tot die sistematiese uitsluiting van kinders in broodnodige intervensionele navorsing, insluitend tuberkulose-middelproewe. Diagnostiese navorsing in PTB in kinders was tot dusver ook swak gestandaardiseer, wat veralgemening en vergelyking van verskillende studiebevindinge moeilik maak. Addisioneel het die onsensitiewe diagnostiese verwysingsstanddaard vooruitgang om nuwe diagnostiese toetse vir kindertuberkulose te ontwikkel, belemmer.

In 'n poging om meer haalbare strategieë te ontwikkel om die mikrobiologiese toetsing te verbeter en te bevorder in kinders met vermoedelike PTB wat in areas woon met 'n hoë TB-las, het ek 'n goed-gekarakteriseerde kohort van kinders wat voorgedoen het met vermoedelike PTB by hospitale, ingesluit in 'n studie. Kinders is deeglik ondersoek, met die gebruik van gestandaardiseerde metodes en met intensiewe monsterversameling vir vloeibare kultuur en molekulêre toetsing met Xpert® MTB/RIF (Xpert). Borkasplate is dubbel geles deur kliniese kenners en gerapporteer op standaard vorms. Alle kinders is opgevolg, onafhanklik van hulle finale diagnose en hul spektrum van TB siekte; kinders met nie-TB verwante siekte is ook goed beskryf. Ek het 'n aantal nuwe diagnostiese strategieë evalueer, insluitende die gebruik van stoelgangmonsters vir die diagnose van PTB met kultuur en Xpert, asook die gebruik van verskillende stoelgangprosesseringsmetodes. Ek het die waarde van gekombineerde respiratoriese monsters evalueer in 'n poging om die diagnostiese opbrengs te verbeter, en om laboratoriumkoste te verminder. Van belang is dat ek 'n konseptuele en kliniese

raamwerk ontwikkel het vir die toekomstige evaluering van nuwe diagnostiese toetse en biomerkers vir die diagnose van PTB in kinders.

Die totale kohort het 608 kinders ingesluit en was verteenwoordigend van die demografie en spektrum van TB in kinders wat in baie hoë-las areas gesien word, waar die meeste kindertuberkulose in jong kinders voorkom. Die mediane ouderdom van die kinders in die kohort was 16.2 maande, met 11.8% van kinders MIV-geïnfekteer. Babas onder 6 maande ouderdom het amper 15% van die kohort uitgemaak. Meer as 20% van kinders het met atipiese kliniese simptome voorgedoen, met 'n soortgelyke prevalensie van akute simptome in all ouderdomsgroepe en diagnostiese kategorieë. Radiologiese kenmerke wat nie tipies van PTB is nie, was algemeen, en dui op die hoë las van respiratoriese patologie, asook die potensiële atipiese radiologiese voordoening van PTB. Twee-honderd-een-en-tagtig (46.2%) kinders is gediagnoseer met PTB en aan hulle is antituberkulose-behandeling voorgeskryf: 117 (41.6%) is bakteriologies bevestig met Xpert of kultuur, 'n hoë diagnostiese opbrengs, as mens in aanmerking neem dat ongeveer 50% van kinders met PTB, nie erge vorms van PTB gehad het nie. Verder is 20/327 (6.6%) kinders wat aanvanklik as simptomatieses kontroles geklassifiseer was, op antituberkulose behandeling begin, binne twee maande nadat hulle in die studie ingesluit is, as gevolg van swak kliniese respons of positiewe bakteriologiese toetsing wat aanvanklik of gedurende opvolgbesoeke, gedoen is. Hierdie data beklemtoon die waarde van noukeurige monstervesameling, insluitend verskillende tipes monsters en diagnostiese toetse, en die belang van kliniese opvolg van alle kinders met vermoedelike TB.

'n Onverwagte hoë proporsie van jong babas onder die ouderdom van 6 maande, het erge vorms van PTB gehad, insluitende kaviteite, wat geassosieer was met 'n hoë bakterie lading en smeer-positiwiteit. Verder was jong babas en MIV-geïnfekteerde

kinders hoë-risiko-groepe vir gedissemineerde TB. Hierdie data beklemtoon die dringende prioritering wat gegee moet word aan die ontwikkeling van toepaslike diagnostiese toetse wat M.tb vinning kan diagnoseer en kwantifiseer in die vroeë stadiums van siekte, voordat TB vinnig na na erge vorms van siekte progresseer in kinders. Versigtige aandag moet ook gegee word aan infeksiebeheer met die ondersoek en hantering van TB in kinders, insluitende jong babas met vermoedelike TB.

Ek het getoon dat stoelgang monsters nuttig is om M.tb te bevestig met Xpert in kinders met erge vorms van TB longsiekte, veral in kinders met longkaviteite, in wie stoelgang toetse TB bevestig het in 45% van kinders wat positiewe respiratoriese monsters gehad het. 'n Nuwe sentrifugeer-vrye prosesseermetode vir stoelgangmonsters (stoelgang toets stel) het soortgelyke resultate getoon as die meer intensiewe sentrifuguurmetodes wat ek inisieël ondersoek het. Hierdie nuwe benadering sou gebruik kon word met meer sensitiewe molekulêre toetse in die toekoms om die bevestiging van longtuberkulose in kinders deur stoelgangtoetsing, te verbeter. Aan die ander kant, was die gebruik van stoelgangkultuur van min waarde om M.tb te diagnoseer, primêr vanweë die hoë kontaminasierisiko (>41% van stoelgangkulture), met die gebruik van die huidige standaard NALC-NaOH (1.25%) dekontaminasie-protokolle.

Ter slotsom, het ek ook getoon dat in kinders wat nie spontaan sputum kon uithoes nie, die toetsing van tot 3 respiratoriese monsters per kind van verskeie tipes (maagsappe, geïnduseerde sputum en nasofaringeale aspirate) in kombinasie, 'n soortgelyke opbrengs gehad het met Xpert en kultuur, as monsters wat individueel getoets was op die drie monsters. In gepaarde analise, het gekombineerde monsters 'n betekenisvolle hoër opbrengs gehad as geïnduseerde sputum en nasofaringeale aspirate op hul eie, maar het 'n soortgelyke opbrengs gehad as 'n enkele maagsap (86.5% vs. 74.4%, onderskeidelik,  $p=0.46$ ). Die algehele opbrengs van die drie individuele

respiratoriese monsters wat invidiueel getoets is, was 86% van alle bevestigde gevalle, soortgelyk aan die algemene opbrengs van gekombineerde monsters. Hierdie resultate ondersteun die aansienlike diagnostiese waarde van 'n enkele maagsap met Xpert en kultuur, en van die nut van 'n diagnostiese "voorladingstrategie" met verskeie monsters wat op een dag versamel word, om die haalbaarheid van monsterversameling vir die diagnose van PTB in jong kinders te verbeter.

Gedurende hierdie kohortstudie, het ek omvattende opvolgdata versamel wat die respons op antituberkulose-behandeling, asook die kliniese verbetering in kinders wat nooit antituberkulose behandeling ontvang het nie (simptomatiese kontroles), beskryf, tot en met 6 maande opvolg. Hierdie data sal verder analiseer word om die diagnostiese waarde van die huidige voorgestelde kliniese gevaldefinisies te evalueer, asook die diagnostiese waarde van kliniese en ander opvolgondersoeke. Kinders wat inisieël as simptomatiese kontroles voorgedoen het met simptome verdag van PTB, sal verder analiseer word om die spektrum van non-tuberkulose-verwante longsiekte in kinders in areas met 'n hoë las van TB te karakteriseer. Ek het ook 'n versameling van goed-gekarakteriseerde monsters insluitende blood en urine, vir die toekomstige evaluering van belowende diagnostiese en prognostiese biomerkers vir kindertuberkulose, gevestig.

Ter opsomming, deur hierdie navorsing het ek nuwe data versamel oor die gebruik van nuwe toetse en diagnostiese strategieë vir die diagnose van kinderlongtuberkulose in MIV-geïnfekteerde en on geïnfekteerde kinders in hoë las-tuberkulose areas. Ek het hierdie data geanaliseer met betrekking tot relevante kliniese en laboratorium-eienskappe sodat ek spesifieke aanbevelings kon maak rondom die mees toepaslike plasing van hierdie strategieë, met inagneming van beidie die teikenpopulasies en verskillende vlakke van gesondheidsorg. Ek kon dit behaal deur 'n goed-ontwerpte studie uit te voer, in 'n goed-gekarakteriseerde kohort en deur

omvattend verslag te doen oor alle aspekte van die studie, insluitende nie-evalueerbare resultate, en komplekse kliniese scenarios. Al hierdie aspekte moet in ag geneem word met die beplanning, analise, en verslaggewing van toekomstige studies om PTB in kinders te diagnoseer. Ek het 'n streng raamwerk gebou vir die evaluering van toekomstige nuwe diagnostiese strategieë vir PTB in kinders, en het verskeie areas identifiseer wat verdere navorsing en intervensie benodig.

## Declaration

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This dissertation includes four original papers published in peer reviewed journals (Chapter 6 to 9) and two unpublished chapters (Chapters 4 and 5). The development and writing of the papers (published and unpublished) were the principal responsibility of myself. For the paper in chapter 7 (“Evaluation of a novel centrifugation-free processing method for the detection of Mycobacterium tuberculosis from stool in young children using the Xpert MTB/RIF assay”), I was joint first author with L. Scott. For each of the other papers, I was the sole first author and my role in each is declared in the relevant chapters. The role and extent of the contributions of each of the other co-authors was disclosed to the journal to which the paper was submitted.

Signed

A handwritten signature in black ink, consisting of a stylized, cursive name.

✍

Date: 31 July 2018

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## Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
AMIK	Amikacin
ANOVA	Analysis of Variance
ART	Antiretroviral therapy
BAL	Broncho-alveolar lavage
BCG	Bacille Calmette Guérin
BCH	Brooklyn Chest Hospital
CFP-10	Culture Filtrate Protein-10
CI	Confidence interval
Ct	Cycle threshold
CXR	Chest radiograph
DNA	Deoxyribonucleic acid
DR-TB	Drug-resistant tuberculosis
DST	Drug susceptibility testing
DS-TB	Drug-susceptible tuberculosis
ELISA	Enzyme-linked immunosorbent assay
EPTB	Extrapulmonary tuberculosis
ESAT-6	Early secretory antigenic target
ESP	Expectorated sputum
FDA	Food and Drug Administration
FIND	Foundation for Innovative New Diagnostics
FNAB	Fine-needle aspiration biopsy
GA	Gastric aspirate

GL	Gastric lavage
HFAZ	Height/Length-for-age Z-score
HIV	Human Immune-deficiency Virus
IFN- $\gamma$	Interferon-gamma
IGRA	Interferon-gamma release assay
INH	Isoniazid
IP-10	Interferon-gamma-induced-protein-1
IPT	Isoniazid preventive therapy
IQR	Inter-quartile range
IS	Induced sputum
KBH	Karl Bremer Hospital
LAM	Lipoarabinomannan
LJ	Löwenstein-Jensen
LPA	Line probe assay
LTFU	Lost to follow-up
MDR-TB	Multi-drug resistant tuberculosis
MGIT	Mycobacteria Growth Indicator Tube
MODS	Microscopic-observation drug-susceptibility
NAAT	Nucleic-acid amplification tests
NALC-NaOH	N-acetyl-l-cysteine-sodium hydroxide
NGT	Nasogastric tube
NHLS	National Health Laboratory Service
NIH	National Institutes of Health
NPA	Nasopharyngeal aspirate
NTP	National Tuberculosis Program



OFL	Ofloxacin
OR	Odds ratio
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMTCT	Prevention of Mother-to-child transmission
PPD	Purified protein derivative
PTB	Pulmonary tuberculosis
QFT-GIT	Quantiferon® Gold-In Tube
RIF	Rifampicin
SHINE	Shorter Treatment for Minimal TB in Children
SOP	Standard Operating Procedure
STARD	Standards for Reporting of Diagnostic Accuracy
TB	Tuberculosis
TBH	Tygerberg Hospital
TB-LAMP	TB-loop-mediated isothermal amplification
TST	Tuberculin skin test
TTP	Time to positivity
UK	United Kingdom
USA	United States of America
VL	Viral load
WFAZ	Weight-for-age Z-score
WHO	World Health Organization
Xpert MTB/RIF	GeneXpert <i>Mycobacterium tuberculosis</i> /Rifampicin assay
ZN	Ziehl–Neelsen

## **Dedication**

I dedicate this research to Lourens, for walking beside me all the way.

And to my two mothers, Maria Cristina Ghimenton and Christina Walters, for giving  
up so much so that their children could fly.

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# Chapter 1

## Introduction

### 1.1 Global burden of tuberculosis in adults and children

Tuberculosis (TB) is a leading cause of morbidity and one of the 10 top causes of mortality worldwide <sup>1</sup>, with the World Health Organization (WHO) estimating >10.4 million incident cases and 1.67 million deaths due to TB in 2016 <sup>2</sup>. Children comprised approximately 10% of the global TB burden, with over one million new cases estimated to have occurred in children below 15 years of age during 2016 <sup>2</sup>. However, fewer than 50% of all paediatric cases (434,044; 43%) were reported to National TB Programmes <sup>2</sup>. Furthermore, an estimated 253,000 TB-related deaths occurred in children in 2016, amounting to a case fatality rate of 25% <sup>2</sup>. A recent modelling study estimated 80% of paediatric TB-associated deaths to occur in children below 5 years of age, and calculated that >96% of childhood TB deaths were in children who were not receiving antituberculosis treatment <sup>3</sup>. These statistics are particularly concerning, knowing that TB is both preventable and treatable in children.

### 1.2 Regional and local burden of paediatric TB in Africa

Almost one third of the total paediatric TB case load in 2015 was reported in the WHO African Region: of the 253 000 paediatric estimated TB deaths for that same

year, 102 000 (40.3%) occurred in Africa <sup>2</sup>. In the Western Cape province of South Africa, the estimated overall TB incidence in 2015 was 681/100,000 population <sup>4</sup>. The Cape Town City Health district reported 23,846 newly registered cases of drug-susceptible TB (DS-TB) during 2016, of which 2,217 (9.3%) were in children <15 years (*unpublished data, Cape Town City Health Directorate*). South African mortality data for 2016 place TB among the 5 leading causes of death in children <15 years of age (3.4% of deaths in <15 year olds and 5.1% in 1-5 year olds) <sup>5</sup>.

### **1.3 Pathophysiology and natural history of TB in children**

The pathophysiology of childhood TB is classically conceptualized to involve three key events: 1) exposure to *Mycobacterium tuberculosis* (*M.tb*), 2) *M.tb* infection, and 3) development of active disease <sup>6</sup>. Exposure implies significant contact with a contagious TB source case. In high-TB-burden countries, up to 60% of children <5 years of age with TB have documented household exposure <sup>7,8</sup>. Following exposure to and infection with *M.tb*, containment of *M.tb* is determined by the host immune response <sup>9-12</sup>. The risk of disease progression is inversely related to age, with an immature or inappropriate immune response favouring disease progression <sup>6,13</sup>. Children <2 years of age have the highest risk of rapid progression from asymptomatic infection to uncomplicated TB disease and also to severe forms of TB disease, including TB meningitis and miliary TB, particularly in the first year following infection <sup>6</sup>. The estimated lifetime risk of TB disease is 10-20% in HIV-uninfected children ≤5 years of age with tuberculin sensitivity (i.e. with reactive TST indicative of TB infection) <sup>14</sup>. Impaired cellular immunity due to young age <sup>13</sup>, HIV

infection<sup>15</sup> or malnutrition increase this risk of TB disease progression. HIV-infected children who do not receive antiretroviral therapy (ART) or who have recently been initiated on ART are at high risk of progressing to severe manifestations of TB disease<sup>16,17</sup>, and experience higher rates of antituberculosis treatment failure<sup>18</sup>, TB recurrence<sup>19</sup> and death<sup>16,18,20,21</sup>. Although primary TB is a relatively benign form of TB, paediatric TB encompasses a wide spectrum of disease manifestations, with many disease forms resulting in substantial morbidity and mortality, seen in severe pulmonary disease, disseminated TB (miliary and central nervous system TB), abdominal and spinal TB.

#### **1.4 Diagnostic challenges in paediatric pulmonary TB**

The earliest and most common manifestation of TB disease in children is intrathoracic mediastinal lymphadenopathy, which, although considered extrapulmonary TB in adults, is commonly referred to as pulmonary in children. The term pulmonary TB (PTB) in this dissertation encompasses all intrathoracic manifestations of TB commonly observed in children. Despite occurring in >75% of children treated for TB<sup>22,23</sup>, PTB remains challenging to clinically diagnose and microbiologically confirm in children, partly due to its non-specific presentation, and partly due to the low sensitivity of currently available confirmatory tests to detect paucibacillary PTB, including molecular tests and mycobacterial culture (the current gold standard of diagnosis), which typically confirms TB in <40% of children<sup>24,25</sup>. These challenges are most pronounced in young children, who bear the greatest burden of disease due to their immunological immaturity and their risk of rapid

progression to severe and disseminated TB after infection with *M.tb* <sup>6,26-28</sup>. Young children are unable to spontaneously produce sputum for microbiological evaluation: resources, in the form of trained health care workers, equipment and in-patient facilities, are required to collect adequate respiratory specimens from young children and infants. Resource-intensive specimen collection coupled with diagnostic tests which have low sensitivity currently constitute barriers preventing timely and accurate detection of TB in children.

Treatment outcomes in children with PTB are typically excellent with early diagnosis and appropriate treatment <sup>29,30</sup>. However, the discrepancy between estimated paediatric caseloads and case notifications <sup>2,31</sup>, combined with published post-mortem data documenting that children in Africa are still frequently dying with undiagnosed TB <sup>32-34</sup>, suggest that diagnostic delay and missed diagnosis remain common in children with TB living in resource-limited settings, ultimately resulting in poor outcomes <sup>35-37</sup>. Although socio-economic and health system factors contribute to diagnostic delay <sup>38-40</sup>, the limited utility and low feasibility of currently available diagnostic approaches to detect PTB in children represent critical barriers preventing access to timely diagnosis and treatment.

From a public health perspective, our current inability to appropriately diagnose children with PTB contributes to under-treatment, under-reporting of cases, inaccurate estimates of the burden of TB disease in children, and insufficient resources made available to combat one of the leading causes of illness and death in children globally <sup>1</sup>. These challenges also present an obstacle to achieving target 3.2 of the United Nations Sustainable Development Goals, which is to end preventable

deaths of newborns and children under 5 years of age by 2030 <sup>41</sup>. From an individual patient perspective, while missed and delayed diagnoses of PTB in children result in more severe disease and poorer clinical outcomes <sup>6,32,42</sup>, over-treatment also exposes young children to unnecessary and potentially toxic drugs and the risk of side effects, while placing the additional burden on families and health care systems posed by antituberculosis treatment.

While improving the detection of TB in children is a priority, the ability to microbiologically confirm *M.tb* is increasingly being recognized as critical. The current burden of childhood TB and ongoing diagnostic challenges experienced in routine care, are evidence that the widely used diagnostic algorithms based on symptoms and non-microbiological investigations are inadequate to either rule out or confirm PTB disease in children <sup>43,44</sup>. In low-resource settings, where the burden of other respiratory infections <sup>45,46</sup>, malnutrition <sup>47-50</sup> and HIV infection <sup>51-53</sup> in children is high, clinical algorithms are unable to distinguish between these common childhood conditions. Misdiagnosis of any of these illnesses, due to insensitive and non-specific tools for diagnosis, has serious consequences for the health of children, especially those living in the poorest settings. Furthermore, with the increasing global burden of drug-resistant TB (DR-TB), children are increasingly also being infected with drug-resistant *M.tb* strains <sup>54-56</sup>. Appropriate treatment requires identification of the organism and its drug-susceptibility status. It is therefore of paramount importance to develop and investigate strategies that will improve and promote microbiological testing of children with suspected TB living in high TB-burden settings. Diagnostic strategies and approaches should be feasible to implement in

such settings, while the scientific methods of evaluation of these approaches should be sufficiently rigorous to be generalizable to most settings where children with TB and TB/HIV live.

## **1.5 Problem statement and scope of this doctoral dissertation**

At the time when I formulated my doctoral research, I identified the following aspects as the most relevant problems with currently available diagnostic approaches for paediatric PTB:

1. Microbiological tests, including smear microscopy, molecular tests and culture, have low sensitivity to detect most children with PTB, who typically have paucibacillary disease (low organism load). In order to improve microbiological detection, it is therefore usually recommended that multiple specimens be collected. This has implications for both the feasibility and the cost of investigations in resource limited settings: given that most childhood PTB occurs in children below 5 years of age, who cannot readily expectorate sputum samples, the collection of appropriate clinical (respiratory) specimens for diagnostic testing requires trained health care workers, equipment and adequate clinical and laboratory facilities resulting in significant resource requirements.
2. There has been limited research to date on the relationship between the spectrum of PTB disease in children and the performance of microbiological tests. Tests applied indiscriminately produce low numbers of confirmed PTB cases in children and high costs, both of which result in low confidence to



attempt the bacteriological confirmation of PTB in children. This phenomenon is often used to justify lack of health care expenditure on TB diagnostic testing for children in many low-resource settings.

3. As a result, health care workers in resource-limited settings usually rely on clinical algorithms to diagnose TB in children, which have high levels of variability and poor specificity<sup>43,44</sup>. In many settings, a diagnosis of TB is missed or delayed, leading to progression of disease and even death, especially in young children, where the risk of disease progression is the highest<sup>32,57</sup>.
4. To date, there has been limited incentive to develop new diagnostic tests specifically tailored for children, in view of the challenge of not having a reference standard with both high sensitivity and specificity for TB disease, to serve as an adequate comparator. In addition, the perceived low public health priority of paediatric TB (although children contribute at least 10% of the estimated global TB case load), has also been a disincentive to invest in diagnostic tests for children, who typically do not transmit *M.tb*. As a result, new tests are developed for adults and tested on adult populations first, without specific paediatric consideration. Their current modest performance in children results in further lack of confidence to attempt diagnostic confirmation in children with presumed (clinically suspected) TB.
5. Historically, when I started my doctoral research, the data on diagnostic studies for paediatric PTB had much variability, including differences in study methodology, the spectrum of TB disease included and described, and the typical lack of long-term clinical follow-up. Published studies have often been

of variable or poor quality <sup>58-60</sup> with limited internal validity and generalizability. Non-evaluable results, including indeterminate/invalid results and contaminated cultures, are infrequently reported. Combining data, for example in meta-analyses, to inform policy recommendations, is also problematic given differences in study methodology <sup>31,60</sup>.

In 2012, a collaborative international consultation among experts in the field of paediatric TB, supported by the United States National Institutes of Health (NIH), developed consensus recommendations on clinical case definitions for paediatric PTB for application in diagnostic research <sup>61</sup>, and on methodological issues for PTB diagnostic research in children <sup>31</sup>. The aim of these guidance documents was to develop a more standard approach for the evaluation of new diagnostics for paediatric PTB. These clinical case definitions were revised in 2015, following early evaluations from several groups. However, the evidence base for some of the elements included in the case definitions still remained limited, for example the utility and timing of follow-up as a diagnostic criterion to confirm or exclude TB disease. The 2015 NIH PTB case definitions acknowledged that further research was urgently needed, including on the value of follow-up and response to treatment in the diagnosis of PTB in children.

Given the challenges outlined above, and the need identified by the global research community to generate high quality data to inform future development of child-focused diagnostic tools for paediatric PTB, I developed a conceptual framework to illustrate a) the requirements, and b) the relevance and impact of

improved microbiological diagnosis of PTB in children (Figure 1). Within this framework, I identified the following key considerations needing to be addressed for the improved diagnosis of PTB in children:

1. There is an immediate and unmet need to evaluate diagnostic strategies for PTB in children that are feasible and affordable for use in resource-limited settings, using the available resources and validated diagnostic tests (Figure 1; Block C). Consideration should also be given to the most appropriate placement of these strategies (e.g. primary care vs. hospital level), their function within the diagnostic framework (triage vs. replacement vs. confirmatory strategy) and diagnostic algorithms, and to the target patient population with relevant parameters such as age and severity of disease.
2. There is a long-term need to develop new diagnostic tests and strategies that are specifically designed for children, with consideration of their unique and varied spectrum of disease and generally low mycobacterial concentrations in respiratory secretions (Figure 1; Block D).
3. Given the absence of an adequate diagnostic reference standard, the evaluation of new tests or diagnostic biomarkers for paediatric PTB will require well-designed and rigorously implemented studies. Key aspects of such studies would include detailed documentation of the study population and study sample, including eligibility/entry criteria, clinical evaluation and interpretation thereof, specimen collection procedures, including specimen storage and transport, and laboratory techniques. Relevant clinical co-variates should be included and described, including HIV status and immunological

markers, nutritional status, spectrum and severity of disease, and information about other possible clinical diagnoses/ co-morbidities. In addition, these well-characterized prospective paediatric cohorts should include a minimum duration of follow-up, well-defined disease phenotypes, and should include ill (symptomatic) controls. Longer term outcomes are needed to evaluate response to antituberculosis treatment, particularly in children with a clinical TB diagnosis (i.e. those not microbiologically confirmed), or clinical progress in the absence of antituberculosis treatment, in order to verify the initial diagnosis. Symptomatic children who are not initiated on antituberculosis treatment should be followed in the same way as children initially classified as TB cases, to evaluate (or validate) the performance of novel tests and clinical progress over time in this clinically relevant control group. Studies should be reported in sufficient detail and clarity as per Standards for Reporting of Diagnostic Accuracy (STARD) guidelines <sup>62</sup>. Relevant paediatric populations with potential to gain from these diagnostic studies in the future should be included. Young children and infants should be specifically targeted for inclusion in such diagnostic studies, due to their disproportionate burden of TB-related morbidity and mortality, their vulnerability to rapidly progress to severe forms of TB, and the simultaneous diagnostic challenges, which are most prominent in the very young (Figure 1; Blocks A and B).

Improving the diagnosis of PTB in children (including earlier diagnosis and with microbiological confirmation) in clinical settings (Figure 1; Block E) will result in appropriate and more timely treatment of children (Figure 1; Block G), and may

prevent long-term morbidity and mortality, especially in children at the highest risk of TB and severe TB due to young age, HIV infection and malnutrition.

The improved diagnosis of PTB in children is also likely to lead to better reporting (notification) and better estimates of local-level and global burden of paediatric TB (Figure 1; Block F). Through continued research and advocacy, this could result in improved allocation of funding and resources to better prevent, diagnose and treat children from high TB-burden settings (Figure 1; Block G). Better diagnostic tools and biomarkers (Figure 1; Block D) will also allow more children to be included in interventional trials of novel antituberculosis drugs and improved (shorter, more potent, less toxic) regimens (Figure 1; Block I). The framework developed for characterising response to antituberculosis (and other) therapy (Figure 1; Block B) for the evaluation of diagnostic biomarkers which may be more sensitive than current diagnostic tests will in turn also provide an evidence base for evaluating antituberculosis treatment response, and therefore treatment efficacy, in children enrolled in such interventional trials (Figure 1; Block H). More effective, shorter and potentially less toxic treatment (Figure 1; Block J) will also result in better care for children with TB (Figure 1; Block G).

My doctoral research was therefore conceptualised and designed to address some of these aspects and research gaps, using the following methods:

1. A representative cohort of children with suspected PTB was enrolled and followed prospectively using rigorous and standard approaches for clinical evaluation, interpretation of chest radiography, specimen collection, processing and laboratory testing, and for reporting (Figure 1; Blocks A and

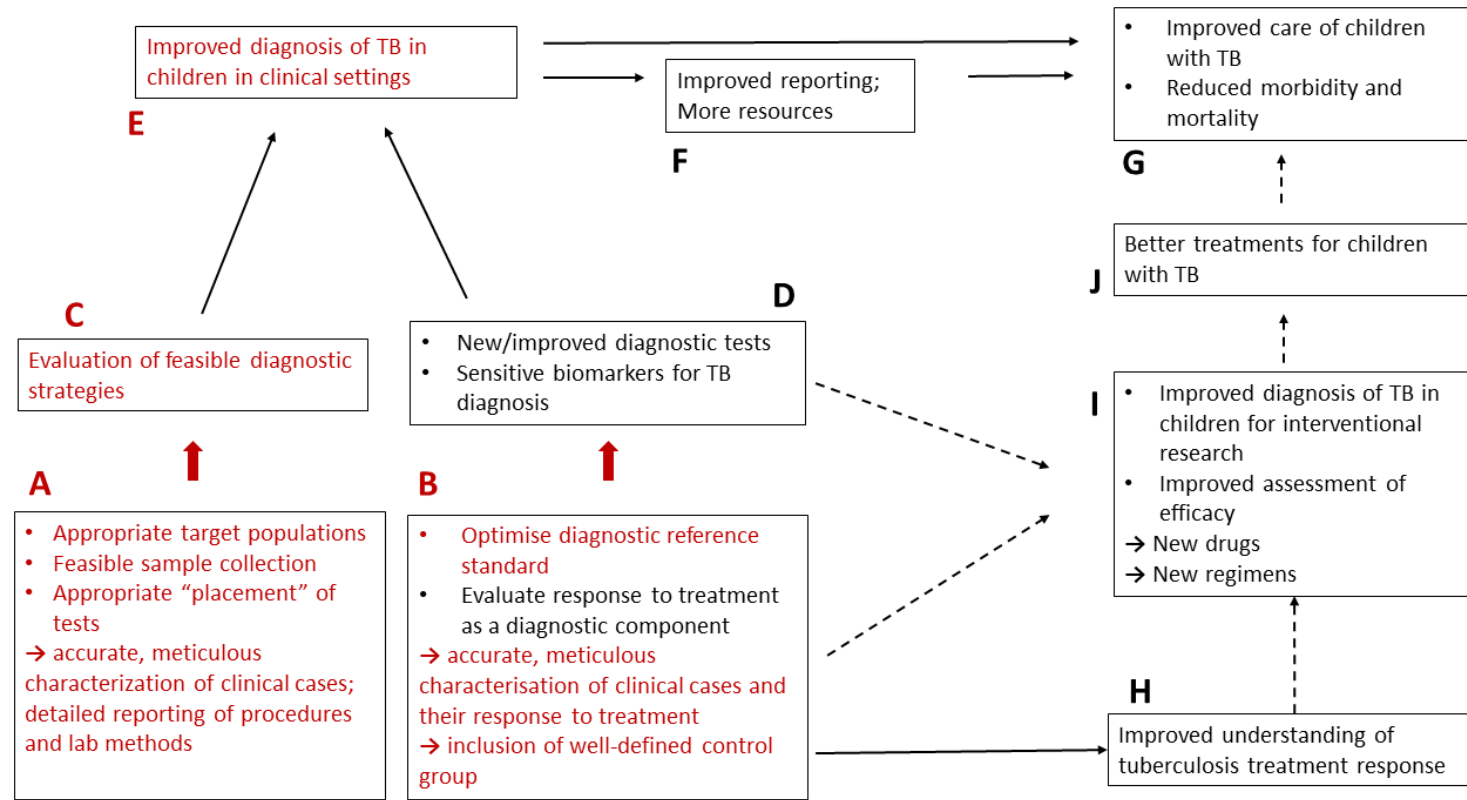
B). This cohort was designed to improve current diagnostic approaches for the bacteriological confirmation of PTB in children. In addition, the study also systematically followed the group of symptomatic controls (“non-TB cases”) as well as TB cases using identical methods, and documented clinical progress using standard approaches. I decided to focus on PTB (rather than extra-pulmonary TB - EPTB) because PTB is the most common form of TB in children, and while it is the most challenging to diagnose, especially in young children, it is also the form most likely to be confirmed microbiologically (with the exception of peripheral TB lymphadenitis - a relatively benign TB manifestation which is readily confirmed through microbiological testing from fine-needle aspiration biopsy; FNAB).

2. In this cohort, I used the available diagnostic platforms at the time, including liquid culture and the Xpert MTB/RIF assay (Xpert; Cepheid, Sunnyvale, CA, USA), to rigorously evaluate novel and potentially more feasible diagnostic strategies for use in resource-limited settings (Figure 1; Block C). Specifically, I evaluated:
  - a. The potential of stool as a child-friendly, readily available specimen to use for the diagnosis of children with suspected PTB, for detection of *M.tb* using Xpert and culture.
  - b. Specimen pooling as a potentially more sensitive and cost-effective strategy using multiple respiratory specimens.
3. I evaluated the relationship between important clinical co-variables, frequently not well-characterized in many paediatric TB diagnostic studies, e.g. the

spectrum and severity of PTB disease, and the bacteriological yield of the different diagnostic approaches, including in specific groups of interest (e.g. HIV-infected children, young infants, those with higher bacillary burden), in order to make relevant, evidence-based recommendations on the utility of the various tested strategies for different clinical and epidemiological scenarios (Figure 1; Block A).

The scope of my doctoral research does not include the detailed analysis of long-term treatment response (Figure 1; Block F), or the development or evaluation of novel biomarkers (Figure 1; Block D). However, in addition to my research questions described above, I wanted to develop a rigorous scientific framework for future work in this field, and to establish relevant cohorts and methods for future work in this area (Figure 1; Blocks A and B). Novel diagnostic approaches which do not rely on the direct detection of the organism are clearly needed, but are beyond the scope of this doctoral dissertation. However, to address this additional important need, I followed all participants to 6 months regardless of treatment status, and established a well-characterised bio-repository of clinical specimens, including blood, urine and stool, for future evaluation of diagnostic and prognostic biomarkers for childhood TB.

## 1.6 Conceptual framework



**Figure 1.1. Conceptual framework for the relevance of diagnosis of paediatric pulmonary (intrathoracic) tuberculosis**

*In red font: Aspects addressed in this doctoral dissertation.*

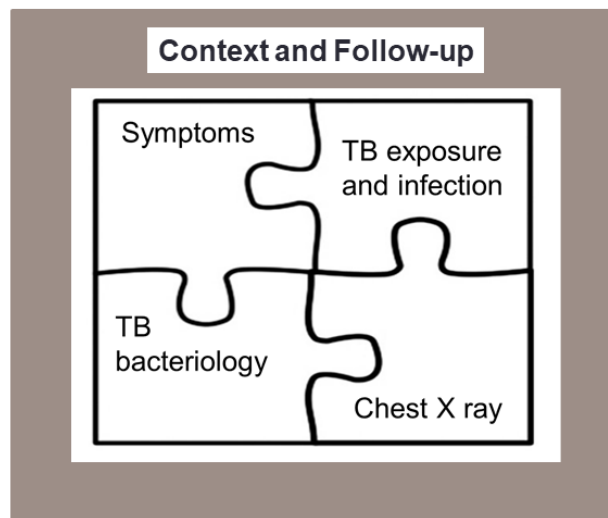


## Chapter 2

### Literature Review

#### 2.1 Diagnosis of paediatric TB: the traditional approach

The diagnosis of PTB in children may be conceptualised as comprising of four broad elements, similar to a four-piece puzzle: 1) clinical signs and symptoms; 2) evidence of TB exposure or infection; 3) chest radiography (or other imaging) and 4) bacteriological testing (Figure 2.1). The weight of these elements may vary within a specific context (e.g. active vs. passive case finding; high vs. low TB-incidence settings, clinical presentation) and their value is supported by clinical follow-up (and repeated investigation if clinically relevant).



**Figure 2.1. The diagnostic puzzle approach for paediatric PTB**

When all four elements are present and are pieced together, they create a convincing image of the “real state” i.e. of true PTB disease. However, frequently, not all four elements are present or available. Within the appropriate context, and if held together by evidence of satisfactory response to treatment at follow-up, two or three elements in routine clinical care are usually considered to be sufficient evidence that the imperfect image generated is close enough to the true disease state. Traditionally, the first three elements (excluding bacteriology) have been considered sufficient to diagnose PTB in children in routine care by TB control programmes in high TB-burden settings, while the bacteriology component has been mostly lacking due to the challenges in achieving microbiological confirmation of *M.tb* in young children, as outlined in Chapter 1. The limitations of this approach have had serious consequences for the health of children living in these settings, by preventing meaningful progress in the field of PTB diagnosis specifically aimed at young children. Relying on the presence of the three non-bacteriological elements may miss PTB in the early stages of disease, which may result in rapid disease progression, especially in young and immune-compromised children. On the other hand, treating children based only on the three elements without bacteriological confirmation may result in missed diagnosis of other serious illnesses which mimic PTB and are especially common in high TB-burden, resource-limited settings; or may result in children being inappropriately treated in the case of undetected drug-resistance.

When I embarked on my doctoral research in 2012, the limitations of non-bacteriological diagnosis of paediatric PTB were increasingly being appreciated <sup>63,64</sup>.

However, the bacteriological component of diagnosis of PTB, specifically for children, was still under-researched.

I present a brief evidence-based outline of the limitations of non-bacteriological diagnostic approaches, and the current state of organism-based diagnosis of paediatric PTB as it was in 2012.

## **2.2 Non-bacteriological diagnosis of paediatric PTB**

### **2.2.1 Clinical presentation**

PTB in children frequently presents with non-specific symptoms such as poor growth, prolonged fever and lethargy<sup>65,66</sup>. Prolonged cough, despite being an organ-specific symptom, is commonly reported with other infectious and non-infectious conditions in children from resource-limited settings<sup>66,67</sup>, including HIV<sup>68,69</sup>. A simple approach using a combination of well-defined symptoms including persistent unremitting cough, documented weight loss over a period of three months before presentation and reported fatigue achieved a sensitivity of 82.3% and specificity 90.2% for diagnosing clinical and confirmed TB in HIV-uninfected children >3 years of age. However, performance was poor in younger children and in the presence of HIV infection<sup>68</sup>. In HIV-infected children not yet on ART or with delayed ART initiation, chronic symptoms are common, and as a result, the approach had low specificity. The low sensitivity observed in young children was ascribed to the more acute and atypical presentation which has been reported in the literature in infants<sup>70,71</sup>.

### 2.2.2 TB exposure

In children, a history of recent exposure to *M.tb* from an infectious (usually adult) source case, particularly if the source case is the child's primary caregiver, is important, especially in young children, who have limited mobility outside of the household. South African studies have shown that a history of TB exposure was more useful than chronic symptoms to differentiate between children with TB and those with other chronic conditions <sup>67</sup>, and that not enquiring about household exposure was a reason for diagnostic delay in young children presenting with non-specific symptoms <sup>72</sup>. In South Africa, a TB source case is identified in approximately 50% of children presenting with a clinical suspicion of PTB <sup>73,74</sup>. Precise quantification of the intensity, frequency and number of TB exposures, as well as the infectiousness of the source case(s) was useful in developing a contact score which strongly correlated with the risk of TB infection in children under 6 years of age living in high TB-burden urban communities in Cape Town <sup>74</sup>. In areas where tests of infection may not be available, this score could be used as a surrogate of infection, and is more sensitive than a simple binary approach to TB exposure. In high-TB burden regions, however, transmission frequently occurs outside the household and is more difficult to verify <sup>75</sup>. In addition, children may be exposed to more than one adult with TB, and possibly to *M.tb* strains with different drug susceptibility patterns <sup>76</sup>, therefore complicating treatment decisions for the child, in the absence of microbiologic confirmation.

### 2.2.3 Tests of *M.tb* infection

Evidence of TB infection is not equivalent to active TB disease, but it provides valuable information of possible causation. This is particularly useful in young

children, who are at high risk of progression to active TB disease within the first year following infection with *M.tb* <sup>6</sup>. In low TB-incidence settings, TB infection in children of all ages is an important component of a clinical diagnosis of PTB. However, in older children from high TB-burden areas, TB infection status may be less useful as a diagnostic tool, as children may have been infected long before the current presentation, compared to young children, who may have had more recent exposure. Two types of tests of infection are mostly used, and endorsed by the WHO <sup>77</sup>: both have advantages and limitations. Most notably, neither can distinguish TB infection from active disease and both are less reliable in the presence of immune compromise, as may occur in infants, in malnourished children and with HIV infection <sup>78,79</sup>. HIV-infected children are both at the highest risk of rapid TB disease progression, and are also the group where a diagnosis of PTB is the most challenging <sup>17,50,80,81</sup>.

a. Tuberculin skin test (TST)

The TST most commonly used is the Mantoux skin test. The procedure for TST placement is readily performed by primary care nurses, through intradermal injection of 5 units of purified protein derivative (PPD) of *M.tb*. The resulting induration is measured using skin callipers 48-72 hours after administration. The reaction is due to a T-cell delayed-type hypersensitivity response to mycobacterial antigens: previously sensitized T-cells are recruited to the site of injection where they release cytokines which induce a local inflammatory reaction <sup>82</sup>. As a result, any cause of defective cellular immunity (including malnutrition, infancy, HIV-infection and severe viral illness such as measles) may result in anergy to TST and false-negative results <sup>83</sup>. Conversely, cross-reaction may occur with a number of environmental

mycobacteria and with the bacille Calmette-Guerín (BCG) vaccine, which is routinely administered at birth to infants living in high TB-burden settings. Although BCG cross-reactivity wanes over time, the diagnostic utility of the TST in vaccinated infants below 6 months of age is limited in settings with high BCG coverage, given reduced specificity. In summary, although TST is useful if positive in young children beyond the neonatal period, it cannot be used to rule out *M.tb* infection in children who are most susceptible to *M.tb* infection and disease.

b. Interferon-gamma release assays (IGRAs)

IGRAs are blood-based assays which measure interferon gamma production (IFN- $\gamma$ ) by circulating lymphocytes after they have been incubated with relatively *M.tb*-specific antigens [culture filtrate protein-10 (CFP-10) and early secretory antigenic target-6 (ESAT-6), with or without TB10.4). Two IGRAs approved by the U.S. Food and Drug Administration (FDA) are commercially available: the QuantiFERON®-TB Gold In-Tube test (QFT-GIT) and the T-SPOT®.TB test (T-Spot). IGRAs' main advantage over TST is better specificity for *M.tb* infection, specifically in the presence of BCG vaccination<sup>84,85</sup>. However, their sensitivity is similar to TST<sup>85-87</sup>, and high rates of indeterminate results have been reported in children with compromised immune function<sup>88,89</sup>. In addition, their high cost currently limits their uptake in resource-limited settings. One study from South Africa reported better performance of IGRA vs. TST to detect *M.tb* infection in HIV-infected children<sup>90</sup>, but the need for more data in this population was emphasized.

#### 2.2.4 Chest radiography

The chest X-ray (CXR) is widely used to diagnose TB in children in spite of its many limitations. Radiological classifications have been proposed, summarising the most common manifestations of paediatric PTB<sup>91,92</sup>. However, the limitations of chest radiology are several, and include difficulties obtaining a technically acceptable image, which requires experienced and well-trained radiographers especially when dealing with young children. Second, the interpretation of what constitutes pathology and the type of pathology seen are both prone to substantial variability, and intra- and inter-observer agreement are poor<sup>93,94</sup>. Finally, the typical radiological manifestations of PTB are not specific for disease caused by *M.tb*; conversely, children with microbiologically confirmed PTB frequently display non-typical, and even normal, radiology<sup>66,95,96</sup>. The most common radiological manifestation of PTB in children is enlargement of mediastinal lymph nodes<sup>73,91</sup>. However, the interpretation of perihilar shadows is frequently subjective, even with skilled readers. Several other limitations with chest radiography include its high cost and limited availability in resource-limited settings and its inability to distinguish between DR-TB and DS-TB.

Despite these limitations, chest radiography may be useful to categorise the severity of pulmonary disease, which may have diagnostic and prognostic implications. Data from South African children with typical radiological manifestations of PTB have demonstrated the relationship between radiological extent of PTB disease in children and bacteriological yield<sup>97,98</sup>: bacteriologic confirmation was achieved in up to 80% of children with extensive and severe lung disease, e.g. TB bronchopneumonia, higher than what is typically reported in children

with PTB. Recently, a comprehensive classification of disease severity for paediatric PTB was developed, allowing for categorization of TB disease based on pathophysiologic, imaging (CXR, computerized tomography and bronchoscopy), bacteriological, and clinical data <sup>99</sup>. This approach allows for the more accurate classification of disease spectrum and severity of TB, including PTB, in children. Evaluation of the feasibility and utility of this classification as a tool to stratify children into diagnostic and prognostic groups was identified as an important future research consideration.

### **2.2.5 Clinical algorithms**

Numerous clinical diagnostic algorithms and scoring systems have been developed, specifically for use in settings with limited capacity for microbiologic confirmation of TB in children <sup>43,44,100</sup>. These algorithms bring together the various elements of clinical diagnosis, and allocate categories based on risk/likelihood of active TB. Although widely used, they are generally poorly validated, and have shown variable performance. Most have limited generalizability beyond the settings for which they were originally designed <sup>43,44</sup> and have not been adapted for inclusion of HIV-infected children. Importantly, these algorithms do not incorporate the increasingly recognised less typical presentations of PTB in children from different clinical, geographical and epidemiological settings.



## 2.3 The microbiological diagnosis of TB in children

Organism-based diagnosis of *M.tb* includes methods which require the presence of whole bacilli for identification, namely smear microscopy, culture and nucleic-acid amplification tests (NAAT), and methods which are able to detect components of the *M.tb* bacillus, such as urine lipoarabinomannan (uLAM) (a cell wall component). The culture methods and NAAT for which paediatric data were available at the time the dissertation was undertaken or which were identified as having potential utility for paediatric PTB diagnosis, are briefly reviewed.

### 2.3.1 Specimen collection

Tests which require whole organisms for detection are typically applied to sputum and other respiratory specimens. This is the first barrier to microbiological confirmation of PTB in children, as young children (typically <5 years of age) require assisted production of sputum by trained health care personnel. In resource-limited settings, bacteriological confirmation, if attempted, usually only occurs in health care facilities such as hospitals accustomed to collecting respiratory specimens from young children. However, although the collection of respiratory specimens from young children is perceived as difficult and resource-intensive, data show that gastric aspiration could feasibly be implemented in a variety of clinical settings with little impact on bacteriological yield <sup>101,102</sup>. It was however recognised that improving children's access to timely diagnosis would require not only more sensitive tests, but also specimens that could be more feasibly collected in the most resource constrained settings <sup>103</sup>.

In addition to gastric aspiration, several alternative respiratory specimen collection techniques have been proposed or revisited, including sputum induction (IS), nasopharyngeal aspiration (NPA), string test and stool collection.

**Gastric aspirates (GA)** and **gastric lavage (GL)** have traditionally been used since pre-chemotherapy times to collect swallowed sputum from young children who cannot spontaneously expectorate <sup>104</sup>. Although GA and GL are frequently used interchangeably, lavage implies dilution of gastric secretions through the instillation of variable amounts of sterile saline or water into the child's stomach before aspiration, while GA involves aspiration of gastric contents without added liquid. No studies have systematically compared the two techniques; in fact, diagnostic studies in children have often included inadequate details on specimen collection, to allow for adequate appraisal of different methods <sup>58</sup>. GA collection requires a minimum period of fasting (4 hours or overnight), which may be challenging to observe for young infants and may require hospitalisation in many settings. Technically, the procedure is relatively simple, as the only requirements are a nasogastric tube (NGT), syringe and specimen jar. Placement of an NGT is a primary healthcare procedure.

**Induced sputum (IS)**, with nasopharyngeal suctioning to collect expectorated secretions in young children and infants, is a feasible and effective alternative to GA collection in hospitalised children investigated for suspected TB <sup>105</sup>. A shorter fasting period is needed (2 hours) than for GA, and the procedure is therefore better suited to out-patient settings. However, careful observation of infection control is needed due to aerosolisation of respiratory secretions during coughing. In addition, sputum induction is contra-indicated in infants and children with severe respiratory distress,

although it has been shown to be safe in very young infants <sup>106</sup>. Considerable resources are needed including equipment (suctioning, oxygen, nebulization).

**Nasopharyngeal aspiration** eliminates the need for nebulisation, but still requires suction equipment and infection control. As a diagnostic specimen, NPA has not performed as well as GA or IS <sup>107-109</sup>.

The **string test** involves swallowing a capsule containing highly absorbent string and leaving the string in the stomach for a period of a few hours to absorb swallowed sputum. In 2012, only feasibility data were available, which indicated that the string test was well tolerated by children >4 years of age. It could not be applied to younger children due to the dangers of possibly swallowing or aspirating the string.

**Stool** collection has not been very actively pursued, due to the limitations of this specimen type both for culture and molecular testing. However, stool is particularly attractive as a sample for TB diagnosis in young children as it is easily obtained and non-invasive. Overgrowth of normal, rapidly-growing stool microbial flora is however problematic for mycobacterial culture. No studies to date have evaluated stool culture for diagnosis in children using liquid medium. Studies on adult stool specimens evaluated different decontamination protocols, with variable results both for *M.tb* detection and contamination <sup>110,111</sup>. Chlorhexidine decontamination followed by incubation in egg-based solid medium showed promising results in adults (sensitivity 54% vs. sputum culture), but the protocols and laboratory materials used are not widely available <sup>112,113</sup>. Data in children are scarce, and include three studies where detection of *M.tb* from stool compared to respiratory specimens was <20% <sup>107,114,115</sup>. Molecular testing of stool specimens is limited by the inhibition

of the polymerase chain reaction (PCR) by the abundance of enzymes in stool, and available data suggest that the sensitivity of NAATs on paediatric stool specimens compared to culture of respiratory specimens is <33%<sup>107,116</sup>.

Older children (typically >5 years of age) who are able to spontaneously expectorate **sputum** should be encouraged to do so, using clear instructions for obtaining an appropriate, non-salivary specimen<sup>117</sup>.

More invasive specimens such as **bronchoalveolar lavage** (BAL) and **transbronchial needle biopsy** have limited applicability in resource-limited settings, but have an important diagnostic value in children with complicated forms of TB or unconfirmed TB with clinical concerns around drug resistance, who are admitted to hospital, if the required technical expertise is available<sup>98,118</sup>.

Finally, **FNAB** of peripheral lymph nodes tested by any of a variety of microbiological techniques has a very high yield in cases of tuberculous adenitis. It has an excellent safety profile and is simple to perform in most clinical settings<sup>119,120</sup>. Given that peripheral lymphadenitis is the most common form of EPTB in children, FNAB is an important specimen in the investigation of children with suspected PTB who present with enlarged peripheral lymph nodes.

**Urine and blood** are seen as highly desirable specimen types to collect from children, as urine is minimally invasive, and blood collection is simply performed by primary care nurses, can be done on demand and does not require sophisticated equipment. However, both specimen types present challenges for use with diagnostic tests. Firstly, they are not organ-specific; therefore organism-based detection from urine and blood has been limited to detection of disseminated or renal TB<sup>121,122</sup>.

Despite the higher risk of disseminated TB in very young children, disseminated TB still only occurs in a small proportion of children with PTB, therefore limiting the applicability of blood testing to a minority of children. Blood is also a complex substratum for molecular diagnosis, as it contains inhibitors which interfere with the PCR. These two child-friendly specimen types have therefore not yet proven useful for diagnosis of PTB with currently available techniques.

**Combined specimen collection strategies.** In children, increasing the number<sup>123-125</sup> and variety<sup>107,108,126</sup> of respiratory specimens collected, improves the overall diagnostic yield (i.e. detection of *M.tb*). Specimen collection over consecutive days may have a higher cumulative yield than same-day collection<sup>108,126</sup>. However, it is less practical in many clinical care settings and more costly if hospitalisation is required for sampling. Pooling respiratory specimens has been suggested as a potentially useful strategy to increase detection of *M.tb*<sup>60</sup>, but no data were available to support this approach.

### 2.3.2 Microbiological tests

PTB in children is typically paucibacillary, implying low bacillary concentrations in respiratory secretions. Currently available diagnostic tests all have limited sensitivity to detect paucibacillary TB, as they were developed for PTB in adults, which is typically characterised by higher bacterial load in sputum. Despite recent advances and improvements in bacteriological tests, their performance in paediatric PTB remains inadequate to detect most childhood PTB, which is clinically diagnosed<sup>127</sup>.

- i. *Sputum smear microscopy.*

Fluorescent microscopy has replaced the Ziehl-Neelsen staining technique as it is more sensitive <sup>128</sup>. However, smear positivity is typically reported to be present in only 10-15% of children with PTB <sup>97,105</sup>. In addition, smear microscopy is not specific for *M.tb* and is seen in disease caused by non-tuberculous mycobacteria or by BCG. Sputum smear microscopy does not distinguish between dead or live bacilli and hence between active or previous TB disease.

ii. *Sputum culture*

Liquid culture (most commonly the Mycobacteria Growth Indicator Tube – MGIT, Beckton-Dickinson, USA) has replaced solid culture in many clinical settings <sup>129</sup>, as it is more sensitive than solid media and has more rapid time to detection <sup>130,131</sup>. Depending on the clinical setting, the patient population and the type and number of specimens collected, culture identifies only approximately 30% of children investigated for PTB <sup>24,67,97,132</sup>. While severe and advanced disease, as often occurs in infants <sup>71</sup> and in children with miliary TB <sup>28,133</sup> is associated with higher rates of culture positivity, this association has not been systematically investigated. In children, due to the low organism load of respiratory specimens, positive cultures typically have a long turnaround time for results (2-6 weeks) <sup>24</sup>. It has been suggested that the decontamination methods used in the laboratory may be too harsh for paediatric paucibacillary specimens and result in loss of viable organisms for culture <sup>134</sup>. In addition, enrichment of the MGIT culture medium was shown to enhance growth of *M.tb* bacilli from GA and other paediatric specimens, resulting in higher detection and shorter time to culture positivity (TTP)<sup>135</sup>. Methods to enhance the

viability and growth of bacilli from culture are highly relevant for the diagnosis of paediatric PTB and should be further evaluated.

Positive cultures require additional tests for the identification of the isolate and drug susceptibility testing (DST), with implications for cost and prolonged time to results. Rapid, affordable antigen-based lateral flow tests which identify *M.tb*-specific MPT64 antigen from cultured isolates, such as the Capilia test <sup>136</sup>, have enabled rapid identification of *M.tb* from culture, but do not provide DST. Line-probe assays (LPA) such as the MTBDR*Plus* (Hain LifeScience, Nehren, Germany) are widely used for rapid DST to isoniazid and rifampicin on positive cultures, but they are less sensitive if applied directly to raw specimens. Direct sputum testing by LPA is only recommended on smear-positive specimens, which is seldom relevant to paediatric PTB. In children who have a high risk of progressing to TB disease and severe forms of TB, waiting for culture confirmation is generally not acceptable, and antituberculosis treatment is commonly prescribed empirically. Although this is not inappropriate practice, it points to the lack of confidence in the current diagnostic tools available for clinical decision-making and further compounds the lack of enthusiasm in attempting to confirm the diagnosis of PTB in children. In addition, for the majority of high TB-burden settings, liquid culture is regarded as expensive and requires a well-equipped laboratory with trained technicians. MGIT culture is also prone to higher rates of contamination than solid culture <sup>137</sup>; contamination is increased further if proper procedure is not followed for the preservation and transport of clinical specimens <sup>138</sup>, which may be problematic in rural and isolated areas.

The microscopic-observation drug-susceptibility (MODS) assay is an alternative culture method, which has comparable sensitivity to MGIT, may be less prone to contamination <sup>139</sup> and provides additional information on drug susceptibility <sup>140-142</sup>. It has been adopted in a small number of clinical settings, including for the diagnosis of paediatric PTB <sup>107</sup>, and is endorsed by the WHO together with liquid culture methods <sup>143</sup>. Although less costly than MGIT culture, this technique still requires relatively complicated laboratory equipment and trained personnel, and global uptake has been relatively limited.

Despite the limitations listed, liquid culture remains the gold standard for diagnosis and confirmation of *M.tb*. Given the problems with low sensitivity in children, evaluation of new diagnostic tests which may have higher sensitivity than the reference standard requires careful consideration to distinguish false positive results from additional detection of true TB diseased cases. In paediatric PTB, this translates to meticulous characterisation of clinical cases and careful follow-up to evaluate response to treatment or clinical progress if treatment is not given.

*iii. Molecular assays (NAAT)*

NAAT are attractive alternatives to culture as they require bacterial DNA rather than live bacilli and therefore may not be as prone to loss of bacilli from transport of specimens or laboratory processes. NAAT are rapid assays, and do not have a requirement for biosafety level 3 laboratories as bacteria are deactivated and lysed before testing and therefore do not pose the same infection risk as for culture. Various types of NAAT are available, including in-house, semi-automated and fully automated assays. Most NAAT use the mycobacterial insertion element IS6110 to



detect *M.tb*. Due to lack of standardisation, in-house NAAT have shown highly variable results for sensitivity and specificity, including on paediatric specimens <sup>144,145</sup> and are not recommended for routine use. Data on commercial NAAT are scarce in children, but suggest lower sensitivity to detect paucibacillary disease states compared to adults <sup>146</sup>.

**The GeneXpert® MTB/RIF system** (Xpert) is a novel fully-automated TB diagnostic test employing real-time NAAT developed by Cepheid, CA, and the Foundation for Innovative New Diagnostics (FIND). It was endorsed in 2010 by the WHO for the rapid diagnosis of TB in adults, particularly in settings with high burden of TB and HIV. Xpert simultaneously identifies 95-98% of rifampicin-resistant *M.tb* strains (a marker of multi-drug resistant TB; MDR-TB), and can provide results in <2 hours <sup>147</sup>. The advantages of the assay, besides its rapid turnover of results and detection of drug resistance, are numerous: it is simple to perform and requires limited operator training, the risk of cross contamination (and hence false positive results) is minimal due to reduced operator handling of the samples (the assay has fully automated sample extraction and in-built amplification controls) and it has a favourable bio-safety profile <sup>148</sup>. Furthermore, it could be used a point-of care test and impact directly on clinical management in real time. An early, large multi-centre demonstration study including patients from the Western Cape Province, South Africa, compared the diagnostic yield of Xpert from sputum with conventional culture methods [solid medium (Löwenstein-Jensen-LJ) and MGIT] in HIV-infected and uninfected adults with suspected PTB. The sensitivity of a single Xpert for detection of culture positive TB was 98.2% for sputum smear-positive TB and 72.5% for smear-

negative TB, with a specificity 99.2%<sup>149</sup>. Sensitivity increased to 85% and 90% with the addition of one and two further tests respectively.

By 2012, Xpert had been evaluated for paediatric PTB diagnosis on IS and NPA<sup>150-152</sup>. Two African hospital-based studies of children with suspected PTB indicated that Xpert had approximately 75% sensitivity compared to MGIT culture, with specificity >98%, using two IS samples per child<sup>150,151</sup>. Sensitivity of Xpert on NPA was similar to IS (65% vs. 71%), although the sensitivity of NPA culture was significantly lower than the sensitivity of culture of IS (97% vs. 71%). These initial data were encouraging regarding the ability of Xpert to detect paucibacillary disease states in children, but specific gaps were identified for the evaluation and application of this novel tool in children. Given the lower sensitivity compared to culture, accurately defining the severity and spectrum of disease would be useful for identifying paediatric patient subpopulations likely to benefit from Xpert testing, including children with DR-TB. Practical considerations such as the performance of the test on different respiratory samples including data on GA, sputum, and less invasive methods such as stool, was also seen as critically important.

#### *iv. Antigen-based tests*

LAM is an immunogenically active glycoprotein constituent of the mycobacterial cell wall, which is released by metabolically active and dying organisms<sup>153</sup>. It undergoes renal filtration and is detectable in the urine. A polyclonal LAM ELISA assay (Clearview® TB ELISA) initially demonstrated significantly better sensitivity (80%) than sputum smear (62%) in culture-positive adults<sup>154</sup>. In addition, LAM was positive in 76.5% of clinical (smear and culture negative) TB cases. Later

studies highlighted the assay's specific utility among severely immune suppressed HIV-infected adults with suspected TB, with sensitivity increasing significantly with decreasing CD4 counts <sup>155-157</sup>. A rapid lateral flow LAM antigen test (Determine® LAM, Alere, Inverness Medical Innovations, Waltham, MA) in 2012 had yet to be evaluated in clinical settings, but the potential for a rapid point-of-care screening test using a non-invasive specimen type was seen as a step towards improving access to diagnostic testing especially in resource-limited settings. There were to date no studies evaluating LAM detection for the diagnosis of PTB in children.

v. *Other tests*

The TB-loop-mediated isothermal amplification (**TB-LAMP**) assay is a rapid manual molecular assay that is visually assessed under ultraviolet light and provides a result within one hour <sup>158</sup>. TB-LAMP has minimal laboratory infrastructure and biosafety requirements and is been evaluated as a rapid, point-of-care test to replace sputum smear microscopy. It does not provide DST results and is less sensitive than culture methods. No paediatric data are available.

The **BlaC-specific fluorogenic** probe is an antigen-based assay with potential for point-of-care application <sup>159</sup>, which uses an *M.tb*-specific enzyme to generate fluorescence which is detectable on a camera such as is built in a regular mobile phone. Very few data on this novel assay were available, none in children.

**Serodiagnostics** to a wide variety of *M.tb* antigens have been evaluated, but extreme variability in sensitivity and specificity have precluded their use. In 2011, the WHO strongly recommended against the use of serological tests for the diagnosis of TB in any patient population <sup>160</sup>.

**Interferon-gamma-induced-protein-10** (IP-10) is an immune cytokine secreted by T-cells in response to stimulation by IFN- $\gamma$ . Studies have demonstrated high levels of IP-10 expressed in both active TB <sup>161</sup> and with *M.tb* infection <sup>162,163</sup>, with potential for improved detection of TB infection in HIV-infected adults <sup>164,165</sup> and in young children <sup>166</sup> compared to IGRAs. However, this assay is not yet commercialised for widespread use, and as a diagnostic test, it is not able to confirm active TB disease.

**Biomarkers and biosignatures of active TB:** there is increasing recognition of the limitations of conventional bacteriology to rapidly detect TB disease in both adults and children. This has resulted in an interest in the discovery and evaluation of potential biomarkers associated with active TB. Biomarkers are defined as measurable biological characteristics which indicate normal biological or pathogenic processes, or pharmacological responses to a therapeutic intervention <sup>167</sup>. Biomarkers can be host- or pathogen-derived and could reflect immunological processes, genetic expression or comprise *M.tb*-specific metabolites. Combinations of biomarkers (biosignatures), in the form of metabolomics, transcriptomics and proteomics, have the potential to increase the sensitivity and specificity of any single biomarker <sup>168</sup>. In 2012, the interest in biomarkers for TB disease was increasing, and research in this field of research was rapidly gaining momentum <sup>169,170</sup>. Several immunological and gene expression profiles associated with active TB, *M.tb* infection, and cure were being evaluated in adults <sup>171-173</sup>, but once again, progress on much-needed biomarkers for paediatric PTB was still limited.

## **2.4 Conclusions and placement of this doctoral research**

From the evidence presented, it is clear that new appropriate diagnostic tests that could rapidly and accurately detect TB disease in children, and simultaneously detect drug resistance, using minimally invasive child-friendly samples, are under-researched but urgently needed in children. The development and application of such tools in children was stated as a priority by the WHO Stop TB Partnership in 2011. However, to date, rigorous research on novel diagnostic tools for childhood TB is scarce and poorly generalizable, due to the lack of standard methodology and inadequate reporting. There is a need to establish a solid framework for the evaluation of new diagnostic strategies, which would include a representative sample population, with broad spectrum of disease manifestations, including non-TB conditions common to children living in high TB-burden settings.

The challenges of establishing such a framework would include the need to recruit a large number of children with suspected TB in order to confirm sufficient numbers to have statistical power for clinically meaningful analysis. In addition, follow-up of the cohort of all recruited children would be an essential element of this evaluation. Retention in resource-limited settings can be challenging and there would be a considerable risk of attrition and loss of data. Effective investigation of children not diagnosed with TB is another important yet challenging aspect in settings with limited resources. This doctoral research aimed to develop such a framework for the comprehensive evaluation of paediatric PTB, to lay a foundation for current and future work on novel diagnostic strategies, including evaluation of specimen

collection methods and strategies, evaluation of novel tests, and the future evaluation of promising biomarkers.

## Chapter 3

### Overview of study cohort and methods

#### 3.1 Study overview

This prospective hospital-based cohort study included the use of rigorous methodology to systematically investigate a cohort of young HIV-infected and uninfected children with suspected PTB, presenting with different spectrum and severity of TB disease manifestations. Participants were recruited consecutively from children routinely presenting to two regional hospitals (one secondary and the other tertiary level), requiring investigation for suspected PTB. Routine investigations including HIV testing, a CXR and TST, were completed in all children as per local standard of care. In addition, at enrolment, multiple representative biological specimens [GA/ expectorated sputum (ESP), IS, NPA, and stool] were collected and analysed by conventional mycobacteriology (smear microscopy and liquid culture), and Xpert.

I evaluated several novel diagnostic strategies using stool specimens and pooled respiratory specimens to improve bacteriological confirmation. Co-variables such as TB disease spectrum and severity and HIV status were evaluated in relation to the bacteriological yield of traditional and novel tools to detect *M.tb*. Serum, urine and stool specimens were also collected for storage and future diagnostic and biomarker work. All children, regardless of a clinical decision to treat by routine

attending clinicians and subsequent antituberculosis treatment initiation, were followed to 2 months for assessment of clinical response, and allocation of a “final TB diagnosis”, based on the current standard consensus diagnostic criteria <sup>174</sup> was allocated, after consideration of bacteriological, clinical and radiographic data at enrolment and of clinical progress and laboratory results at the month 2 visit. TB disease severity was classified using a standard classification <sup>99</sup>. In addition, all children were followed for a minimum of 6 months or through the end of antituberculosis treatment (TB cases). Detailed analyses on long-term outcomes and predictors of antituberculosis treatment response are beyond the scope of this dissertation.

### **3.2 Study setting**

This study was conducted in Cape Town, Western Cape Province, South Africa, where the reported adult TB case notification rate in 2012 was 671/100 000 and 315/100 000 in children <13 years of age (unpublished data, City of Cape Town Health Directorate). The estimated prevalence of HIV among children with culture-confirmed TB was 26% of those tested in 2008 <sup>73</sup>, including in infants <sup>175</sup>. At Tygerberg (TBH; a tertiary provincial referral hospital), over 500 new cases (approximately 150 culture confirmed) of paediatric TB are routinely diagnosed annually <sup>40</sup>. The majority of childhood TB cases (~75%) are intrathoracic <sup>23</sup>, and the prevalence of MDR-TB in children with culture-proven TB at TBH was 8% in 2011<sup>55</sup>.

The study was implemented in TBH and Karl Bremer Hospital (KBH; secondary level hospital). These two hospitals serve as regional referral centres to



the surrounding health sub-districts (Northern, Tygerberg and Eastern districts) in the City of Cape Town, serving over 30% of the metropolitan population (approximately 3.5 million residents). TBH also serves as a tertiary level referral centre for complicated paediatric and MDR-TB. Jointly, the estimated paediatric TB case load for the two hospitals was approximately 600-700 children per year during the period of study implementation. The population served by the two hospitals consists mostly of South African mixed race and black African inhabitants. A large proportion of children from the study population live in poverty, with high rates of unemployment (>23%), over-crowded living conditions and/or informal housing (>18%)<sup>4</sup>.

### **3.3 Study population**

Children aged 0 to 13 years of age (routine paediatric ward admission criteria in the study setting) who routinely presented with suspected PTB were approached for participation. Young children with suspected TB are routinely referred to hospitals for standard investigation including clinical assessment, CXR, TST and collection of respiratory specimens (typically two early morning GA in children <5 years who are unable to expectorate) for mycobacterial culture and DST. Approximately 30% of children initiating treatment for TB in TBH are culture positive in this setting under routine conditions<sup>97,105</sup>. There are no standard guidelines on monitoring response to antituberculosis treatment, which is routinely dispensed to and monitored in most children at the primary clinic level following initial hospital-based diagnosis in the study setting, which follows a decentralized model of TB care,

as is recommended in South Africa. Treatment assessment at the end of the intensive phase of antituberculosis treatment (8 weeks) or at treatment completion (usually 6 months) typically involves symptom evaluation, weight measurement and infrequently, follow-up CXR, although assessment of treatment response is variable, especially in HIV-infected children with co-morbidities. Bacteriological follow-up evaluation is not routine in children with DS-TB, unless there is perceived poor clinical or radiological response.

### **3.4 Recruitment**

The study was conducted from 28 March 2012 to 7 November 2017. The last participant was enrolled on 15 March 2017. Children routinely presenting with symptoms suggestive of intrathoracic TB were consecutively approached for participation and screened for eligibility during week days (Mondays-Fridays) from paediatric wards (inpatient and outpatient/overnight wards). Written informed consent was obtained from the parent(s)/legal guardians of eligible children. Assent was required for children 7 years of age or older who showed adequate understanding (See Appendix B: Standard Operating Procedure-SOP-12 and Appendix C: consent taking). All children consented for enrolment underwent standard systematic investigation (See schedule of evaluations; Table 3.1).

## 3.5 Population and eligibility criteria

### 3.5.1 Inclusion criteria

1. Written consent provided by the parent(s)/legal guardian for study participation, including HIV testing. Assent was required from children 7 years of age and older who showed adequate understanding.
2. Children <13 years of age, weighing >2.5 kg, identified in hospital (inpatient or outpatient) with suspected PTB, including suspected MDR-TB, defined as having  $\geq 1$  of the following symptoms:
  - i. Persistent unremitting cough (or cough significantly worse than usual in child with chronic lung disease including HIV-related) of  $\geq 2$  weeks duration, unresponsive to a course of appropriate antibiotics,
  - ii. Poor growth documented over the preceding 3 months (clear deviation from the child's previous growth trajectory and/or static growth or weight loss in the preceding 3 months; alternatively, weight-for-age Z-score (WFAZ)  $\leq -2$  in children with no previous weight measurements),
  - iii. Persistent unexplained lethargy or reduced playfulness/activity reported by the caregiver,
  - iv. Any duration of cough with  $\geq 1$  of:
    - a) Documented exposure to a known TB source case (regardless of current smear status) OR
    - b) Reactive Mantoux skin test (defined below) OR
    - c) CXR suggestive of TB <sup>91</sup>

- v. In infants 0-60 days, also: neonatal pneumonia unresponsive to broad-spectrum antibiotics/antivirals, or unexplained hepatosplenomegaly, or sepsis-like illness

### **3.5.2 Exclusion criteria**

1. EPTB without concurrent suspected PTB,
2. Receipt of antituberculosis treatment for >1 day before specimen collection for mycobacterial investigation. Children receiving isoniazid preventive therapy (IPT) were not excluded.
3. Severe illness resulting in an unstable clinical condition, where intense sampling would have posed unacceptable risk to the child. Enrolment could be deferred until the child was in a stable condition.
4. An absolute contra-indication to any of the sampling procedures required by the study.
5. Living in remote areas with no ready access to transport for follow-up visits.

### **3.6 Study measures**

Clinical and laboratory investigations were completed in a rigorous standard manner using SOPs. All investigations and measures were undertaken by trained research personnel.

**Table 3.1. Schedule of investigations in all children enrolled**

	Enrolment	Month 1	Month 2
History (including evaluation and quantification of TB exposure)	x	x	x
Clinical examination, including anthropometry	x	x	x
Tuberculin Skin Test (Mantoux)	x		
CXR (antero-posterior and lateral)	x		x
HIV test	x		
Respiratory specimens (smear, Xpert and culture*)	x	x	x
Stool	x		
Evaluation of TB treatment adherence		x	x
Urine (biorepository)	x	x	x
Serum <sup>§</sup> (biorepository)	x		x

*\* 2-4 specimens of at least 2 different types collected over 2 days at enrolment in all children; at follow-up, 2 specimens of 2 types were collected only in TB cases (clinically diagnosed or confirmed); <sup>§</sup>Collection of blood at the 2-month visit was only completed for TB cases; CXR: chest X-ray.*

**Visit windows:** The baseline visit was defined as the date of starting antituberculosis treatment in TB cases, and the date of the enrolment visit for children not diagnosed with TB. For TB cases, a visit up to 14 days before TB treatment initiation was considered to be the baseline visit, in cases where the decision to start antituberculosis treatment may have been delayed. The month 1 visit was allowed a window 15 to 42 days after the baseline visit. The month 2 visit was allowed a window of 43 to 120 days after the baseline visit. Different methods were used to trace participants for follow-up visits (See Appendix B: SOP 9).

**Signs and symptoms** were systematically recorded. Details on the duration and severity of symptoms, TB exposure history, HIV exposure and disease were collected. *M.tb* exposure was ascertained from caregivers using a standard data

instrument to determine the extent of contact between the child and the TB index case in the child's household, or a non-household member in close contact with the child, treated for active TB within the preceding 12 months <sup>74</sup>. The sputum status (smear or culture positive), drug susceptibility pattern and the type of disease (pulmonary vs. extrapulmonary) of any adult TB source case was recorded if known (See Appendix D: SDOC25).

**Symptoms were classified as follows:**

- a) Well-defined symptoms of PTB in children:
- Prolonged unremitting cough: daily cough reported for  $\geq 14$  days.
  - Prolonged fever: fever  $\geq 7$  days, not responsive to antibiotic treatment
  - Prolonged poor feeding in infants or poor appetite in older children for  $\geq 14$  days
  - Unexplained lethargy/ lack of playfulness: child unusually tired/sleepy, not engaging in usual daily activities and play
  - Failure to thrive: clear deviation from the child's previous growth trajectory (evident in the child's growth card) or static growth or weight loss in the preceding 3 months; alternatively, WFAZ  $\leq -2$  in children with no previous weight measurements
- b) Additional symptoms (not included as the "well-defined" symptoms in the literature and NIH case definitions), but reported on in this analysis:
- Prolonged cough: cough reported for  $\geq 14$  days (not daily)

- Prolonged wheeze: wheeze reported for  $\geq 14$  days. The presence/absence of wheeze was only documented in 352 children (the case report form did not include wheeze as a symptom initially)

**Anthropometry** was assessed at each study visit (See Appendix B: SOP 11). Measurements included weight, recumbent length (infants) or height and mid-upper arm circumference using calibrated equipment. UK Z-score charts were used as reference, as they include WFAZ for all ages <sup>176</sup>.

**HIV infection** and measures of immune function.

The provincial policy for early neonatal testing of HIV-exposed infants: From 2012 to 2015, the 2010 National ART Guidelines were followed <sup>177</sup>, which recommended all HIV-exposed infants to have an HIV DNA PCR test at 6 weeks of age, or earlier in case of severe illness or symptoms suggestive of HIV infection. A confirmatory viral load (VL) test was completed on all HIV PCR-positive infants, with fast-tracking to start ART if HIV infection was confirmed. All breastfed infants testing HIV-negative at 6 weeks received an age-appropriate HIV test (see HIV-testing below) 6 weeks or more after cessation of breastfeeding or if clinically indicated during breastfeeding. In 2015, guidelines were updated <sup>178</sup> to recommend HIV PCR testing at birth for all HIV-exposed newborns, with a second confirmatory PCR for infants who tested positive. VL testing as a confirmatory test was discontinued due to the successful implementation of the B+ Prevention of Mother-to-Child Transmission (PMTCT) program, which resulted in undetectable VL even in infected infants due to maternal and neonatal ART. An HIV PCR test was recommended again at 18 weeks for all HIV-negative infants receiving postnatal nevirapine to 12 weeks of age.

HIV testing for children enrolled in the study: In children not documented to be HIV-infected, an HIV DNA PCR test (Roche Cobas AmpliPrep/Cobas TaqMan) was completed at the virology laboratory, National Health Laboratory System (NHLS), TBH, in children <18 months of age, and an HIV ELISA antibody test (Roche HIV Combi PT Elecsys Cobas e100) for children  $\geq$ 18 months. If phlebotomy was insufficient, a rapid HIV test was performed, followed by a confirmatory test if positive. HIV medical records were abstracted in children with known HIV-positive status. In HIV-positive children with no CD4 count or VL, both assays were completed. WHO clinical and immunological staging was determined.

HIV status was defined as follows:

- HIV- uninfected: a child not exposed to HIV perinatally and testing negative on a confirmatory test (see HIV testing above).
- HIV-exposed uninfected: a child exposed to HIV perinatally and testing negative on a confirmatory test
- HIV-infected: a child confirmed to be HIV-infected on a confirmatory test. Not all children had confirmed perinatal HIV exposure, mostly due to absent records in older children and maternal demise.

HIV-infected children who were not receiving ART at the time of enrolment were referred to the Infectious Disease service at TBH or KBH (depending on the recruiting hospital) for expedited ART initiation and HIV care.

**HIV treatment.** During the course of the study, ART guidelines for children changed. Between 2012 and 2015, the 2010 South African National ART guidelines were followed <sup>177</sup>. These recommended ART to all HIV-infected children <1 year of



age (not restricted by clinical stage or CD4 count); children 1-5 years with WHO clinical stage 3 or 4, or CD4 count  $\leq 25\%$  or  $\leq 750$  cells/ $\mu\text{l}$ ; and children  $> 5$  years with WHO clinical stage 3 or 4 or CD4 count  $\leq 350$  cells/ $\mu\text{l}$ . Recommended first-line ART regimen for children  $< 3$  years of age or  $< 10$  kg body weight was abacavir, lamivudine and lopinavir/ ritonavir; and abacavir, lamivudine and efavirenz for older children weighing  $> 10$  kg. Second-line treatment for efavirenz-based regimens was zidovudine, didanosine and lopinavir/ ritonavir; while referral to a specialist centre was recommended in case of failed lopinavir/ritonavir-based regimen. In 2015 these guidelines were updated <sup>178</sup> to expand eligibility to ART for all children  $< 5$  years of age regardless of clinical stage or HIV count, and to children 5-15 years of age with WHO clinical stage 3 or 4 or CD4  $< 500$  cells/ $\mu\text{l}$ . The first-line ART regimen was changed for children  $> 3$  years of age previously exposed to perinatal nevirapine, where lopinavir/ ritonavir replaced efavirenz as the third drug. The second-line regimen after 2015 was zidovudine, lamivudine and lopinavir/ ritonavir for failed efavirenz or nevirapine-based regimen, while a regimen based on genotypic resistance-testing was recommended for failed lopinavir/ritonavir-based regimen. Both guidelines recommended ritonavir boosting of lopinavir/ritonavir-based regimens if a rifampicin-based antituberculosis treatment was co-administered.

**TST:** (Mantoux, 2 Tuberculin Units of PPD RT-23, Statens Serum Institute, Copenhagen). TST was injected intradermally in the volar aspect of the left forearm and read 48-72 hours after placement. A TST reaction was considered positive if the wheal measured  $\geq 10$  mm if HIV-negative and BCG-vaccinated,  $\geq 5$  mm if HIV-positive or not BCG vaccinated. Evidence of BCG vaccination was determined by written

record in the immunization card or evidence of BCG scar in the right deltoid area (see Appendix B: SOP 7).

**Close TB exposure:** Exposure to any identified adult TB source case in the preceding 12 months, where exposure was either within the household; or involved the child's primary caregiver; or occurred for >4 hours per day during the period of exposure.

### **Chest radiographs**

Antero-posterior and lateral films were completed at enrolment and at the month 2 visit, unless clinically indicated before the 2 month follow-up. Films (digital or hard copy) were independently interpreted by two of three paediatric TB experts, blinded to all patient clinical and laboratory data and to the other readers' reports, using a standard form for recording and reporting<sup>91,179</sup> (See Appendix B: SOP 8). Radiographs were considered 'unreadable' if one reader classified the quality as technically unacceptable.

#### a) Radiological diagnostic certainty of TB and observed disease spectrum:

If the quality of the film was acceptable, each reader documented any key radiological features and classified the film according to radiological diagnostic certainty of PTB disease. The following features were classified as being 'typical of TB': presence of soft tissue shadowing suggestive of perihilar or paratracheal lymphadenopathy, airway compression or deviation, expansile pneumonia, a Ghon focus, pleural effusion, miliary infiltrates and cavities (excluding bronchiectasis). The following features, reported without any of the 'typical' features described above, were classified as being 'not typical of TB': alveolar consolidation/collapse (lobar,

segmental or bronchopneumonic), interstitial infiltrates (including perihilar infiltrates), generalised hyperinflation and bronchiectasis.

After independent dual reading of the CXRs, consensus criteria were applied to classify each film as 'normal', 'abnormal, typical of TB' or 'abnormal, not typical of TB'. Where agreement on diagnostic certainty was not reached, a third reader reviewed the film; consensus between 2 of the 3 readers was used to reach a final classification. For reporting patterns of disease, only patterns for which there was complete agreement were reported.

b) Radiological disease severity:

Each CXR was subsequently assessed for radiological severity of disease, using a pragmatic modification of a published classification<sup>99</sup>. Radiographs were classified as reflecting severe disease if there was perfect consensus between 2/2 or 2/3 readers of 1) complicated PTB disease, 2) extensive parenchymal involvement or 3) evidence of dissemination (miliary pattern).

For CXRs typical of TB, severity was defined as either extensive, complicated or disseminated disease:

- Complicated TB was defined as airway compression, expansile pneumonia or cavitating disease (including adult-type PTB disease, cavitating Ghon focus or TB bronchopneumonia).
- Extensive disease was defined as bilateral parenchymal involvement (alveolar consolidation), or involvement of >2 zones of the lung or a total lung surface area considered greater than the surface area of the right upper lobe.
- Disseminated disease was defined as a miliary pattern.

For CXRs not typical of TB (TB cases or symptomatic controls – reviewers were blinded to TB disease status), the extent of parenchymal disease (therefore excluding interstitial patterns and hyperinflation) was documented as it was done for “typical TB” (see above) and complicated disease was defined as the presence of non-TB cavities (e.g. classified as being due to bronchiectasis).

**Additional imaging and other investigations:** results, if routinely completed as part of clinical care (e.g. computed tomography of the chest or brain, abdominal ultrasound and bronchoscopy), were systematically recorded and were incorporated into the final classification to describe the full spectrum of disease. The spectrum of TB disease was recorded based on collated imaging, laboratory and clinical data.

**Respiratory specimen collection** (See Appendix B: SOP 1-3). The study had distinct phases during which different diagnostic strategies were evaluated (See Table 3.2). At a minimum, the specimen collection schedule required one specimen each of GA (in children <5 years of age unable to expectorate spontaneously) or ESP (older children) and IS (with or without nasopharyngeal suctioning depending on the child’s age), collected daily for two consecutive days.

NPAs were introduced in August 2013 (from participant number 101 onwards), in order to evaluate the diagnostic utility of this potentially less invasive specimen type.

If any of the respiratory specimens required by the study had already been collected by the hospital personnel, these were not collected again by the research team, and their bacteriological results were documented. Some children also had

FNAB of peripheral lymph nodes and BAL, collected by the hospital personnel if clinically indicated.

SOPs were followed for all study measures. Specifically, minimum volume requirements for respiratory specimens were prescribed: 5mL for GA, 2mL for ESP, and 1mL for IS and NPA. If greater volumes could be collected, this was encouraged. Specimens were not discarded if the minimum volume could not be achieved.

**Table 3.2. Schedule of collection of respiratory specimens**

	Enrolment		M1	M2
	Day	Day		
	1	2		
<b>Younger children (&lt; 5 years) unable to expectorate sputum</b>				
GA for Smear, Xpert, MGIT	x		x	x
IS for Smear, Xpert, MGIT	x		x	x
NPA for Smear, Xpert, MGIT (for study patients 101 to 540)	x		x	x
Respiratory specimens pooled for Smear, Xpert, MGIT (for study participants 234 to 621)			x	
1. GA				
2. NPA				
3. Sputum induction then suction				
<b>Older children able to expectorate sputum</b>				
Expectorated Sputum early morning after long fast (4hr min) for Smear, Xpert, MGIT	x			x
Expectorated Sputum spot (2h fast) for Smear, Xpert, MGIT	x		x	x
Induced Sputum (IS) for Smear, Xpert, MGIT	x		x	x
Respiratory specimens pooled for Smear, Xpert, MGIT			x	
1. Expectorated Sputum early morning				
2. Expectorated Sputum spot				
3. Sputum induction then expectorated sputum (no suction used)				

*M1: month 1; M2: month 2; GA: gastric aspirate; IS: induced sputum; NPA: nasopharyngeal aspirate; MGIT: Mycobacteria Growth Indicator Tube liquid culture*

**Stool collection.** Stool was collected within 7 days of enrolment. Caregivers were given verbal and written instructions on how to collect stool (Appendix B: SOP 4).

**Specimen handling and transport:** After collection, GA specimens were titrated to neutral pH at the time of collection by the study nurse, using 4% sodium bicarbonate solution and gradated pH strips <sup>180</sup>. All respiratory specimens were kept refrigerated and transported to the laboratory in a cool box within 4 hours of collection. Stool specimens collected at home from children discharged from hospital and stools that could not be processed immediately were stored at 2-8°C for maximum 72 hours before processing.

**Laboratory processing of respiratory specimens:** Respiratory specimens were processed at the NHLS Microbiology Laboratory at TBH according to national standard laboratory procedures (See Appendix B: SOP 13). For digestion/decontamination, NALC-NaOH was used (final NaOH concentration=1.25%), before concentrated fluorescent Auramine-O smear microscopy <sup>128</sup>, Xpert and liquid MGIT (Becton Dickinson, Sparks, MD, USA) culture <sup>181</sup>. The SOP for processing pooled respiratory specimens for the sub-study described in Chapter 9 is detailed in the relevant paper and is provided in Appendix B (SOP 14). Smears were graded according to the WHO/ International Union Against Tuberculosis and Lung Disease classification. Cultures were incubated for up to 42 days. If no growth was observed, cultures were declared negative. For positive cultures, the TTP in days was noted and Ziehl-Neelsen (ZN) stain was performed on the culture. If ZN positive, mycobacterial identification and drug susceptibility for isoniazid and rifampicin were completed using MTBDR*Plus* LPA (Hain LifeScience, Nehren, Germany). The cycle threshold (Ct) values for Xpert-positive specimens were recorded, as well as the semi-quantitative results reported by the laboratory. Rifampicin-resistant strains detected by Xpert or

LPA were transported to the NHLS in Green Point, Cape Town, for phenotypic DST for ofloxacin and amikacin using the agar proportion method. If growth of bacteria/fungi was observed on blood agar plates and/or non-acid-fast bacteria were seen on the ZN smear, the MGIT culture was considered contaminated. Contaminated cultures from respiratory specimens were re-decontaminated and re-incubated once only. Invalid or error Xpert results were repeated if sufficient concentrated specimen was available for second testing.

**Laboratory processing of stool specimens:** Stool specimens were processed by the study laboratory technician. Different stool processing methods were used in different sub-studies and are detailed in chapters 6, 7 and 8, and in Appendix B in SOPs 4, 15 and 16.

**Microbiological confirmation status, for the overall reporting of the cohort:**

- Smear positive: a child with at least one respiratory specimen with a positive smear result (including scanty positive). Smear-positivity was not considered a criterion for microbiologically confirmed TB in the absence of a positive Xpert or culture/LPA result.
- Xpert-positive: a child with at least one respiratory specimen with a positive Xpert result.
- Culture-positive: a child with at least one respiratory specimen with a positive culture result, confirmed to be *M.tb* on LPA.

- Microbiologically negative: a child with all microbiology test results negative (non-positive). Non-positive includes indeterminate/error/invalid results and contaminated cultures.
- Overall microbiologically confirmed TB: *M.tb* confirmed by Xpert or culture/LPA, from any number of respiratory and non-respiratory specimens. Smear positivity in the absence of Xpert or culture confirmation was not regarded as microbiologically confirmed TB.

**Reporting of microbiology results:** microbiology results are defined and reported in detail in the individual diagnostic sub-studies which form part of this dissertation (Chapters 6-9), including all non-valid results (error/invalid Xpert; contaminated cultures; indeterminate DST results). For the description of the clinical cohort (Chapter 4) and the overall microbiology results (Chapter 5), non-valid results were considered non-positive (i.e. analysed as negative) and are not reported separately. The DST results reported in Chapters 4 and 5 are those which were considered as final by the NHLS after investigation and resolution of any potential discrepancies between tests (e.g. between Xpert and LPA rifampicin resistance results), and were used to inform treatment decisions.

**Antituberculosis treatment decision:** The decision to treat for TB was made by the attending clinicians, based on clinical/epidemiological assessment and the results of all laboratory investigations and was not based on research case definitions. All results from study samples, including stool, were available to clinicians. Children were typically prescribed treatment at the time of TB diagnosis in hospital for the duration of their hospital stay. Children diagnosed with DS-TB were discharged to the



local clinic to receive ongoing medication according to South African National TB Programme (SA NTP) guidelines<sup>182</sup>. Referral to care at the local clinic was reinforced through a local initiative linking children diagnosed in hospital to the appropriate primary care clinic<sup>183</sup>. Children with neuro-tuberculosis and miliary TB were typically managed at Brooklyn Hospital for Chest Diseases (BCH), a medium- and long-term TB care facility for patients with complicated forms of TB. Some children with stage 1 TB meningitis were managed as outpatients through a dedicated service established for such patients at TBH in close collaboration with local community TB clinics<sup>184</sup>.

### **Antituberculosis treatment regimens**

DS-TB: A child receiving only drugs used for DSTB. Excludes children who received a combination of DSTB and DRTB regimens, because the diagnosis changed, and children who received therapy for DSTB and additional preventive therapy for DRTB disease. Routine first-line antituberculosis drugs at standard WHO-recommended doses included rifampicin (15 mg/kg/day), isoniazid (10-15 mg/kg/day) and pyrazinamide (35 mg/kg/day), with ethambutol (20 mg/kg/day) added in cases of severe or HIV-associated TB, or with ethionamide (15-20 mg/kg/day) added in cases of neuro-tuberculosis or miliary TB. The local TB clinic was responsible for administering antituberculosis treatment.

DR-TB: A child receiving any drugs used to treat DR-TB (any form, including isoniazid mono-resistance, rifampicin resistance or with any additional resistance), for any duration of time, was classified as having received a DR-TB regimen. Children diagnosed with DR-TB were typically admitted to BCH, at least initially, to receive

tailored antituberculosis treatment based on the child's own DST results, or those of the child's adult source case, according to WHO guidelines<sup>185</sup> and local best practice<sup>186,187</sup>.

**Assessment of TB treatment response:** All children diagnosed with TB who were clinically assessed as responding poorly to treatment at follow-up visits were evaluated for adherence, paradoxical worsening of TB, drug resistance (if not initially confirmed to have DR-TB), and an alternative diagnosis, based on the clinical assessment. As needed, children were referred to sub-specialist services, including paediatric pulmonology, infectious diseases and nutritional rehabilitation at TBH and in the community, for further assessment and investigation.

**Adherence evaluation:** As the study team was not responsible for administering antituberculosis or HIV medications, adherence monitoring was reliant on caregiver reports and collateral information obtained from the clinic where the child was receiving treatment. We used different approaches aimed at identifying non-adherent participants and those who experienced difficulties in administering medication, so as to institute appropriate intervention. At each visit, a TB medication adherence questionnaire was administered to participants by the study research assistant (see Appendix D: SDOC17 Adherence assessment). The questionnaire is based on the Simplified Medication Adherence Questionnaire<sup>188</sup>, and aims to identify non-adherent behaviour as well as quantify the percentage of missed doses by recall. Participants were also asked to score their perceived adherence on a visual analogue scale. In addition, caregivers were encouraged to bring their TB treatment card (routinely issued by local TB clinics) to each study visit. These cards were reviewed

if available and the percentage of missed doses over the previous month was calculated. Caregivers were also questioned on their knowledge of doses and on their experience of giving medication to their child. A cumulative adherence assessment was based on all these measures, and classified as: a) “grave concerns/ non-adherence” if there was evidence of poor adherence/non-attendance at the clinic or serious concerns about intake of medication (severe vomiting/medication refusal), b) “some concerns” if some difficulty administering medication was noted or some doses were missed with valid explanations, or c) “no concerns” if there was no indication of any problems with administering medication. Any concerns raised at the time of the study visit were followed up with a phone call or visit to the relevant local TB clinic for verification. Adherence was supported by a trained adherence counsellor employed by the study, in collaboration with local clinic healthcare workers and treatment supporters.

**Evaluation of clinical response to antituberculosis treatment or clinical progress in the absence of antituberculosis treatment:**

All children, regardless of a decision to treat for TB, were evaluated by the research team at one and two months after “baseline”, which was defined as the date of starting antituberculosis treatment in TB cases, and the date of the enrolment visit for children not diagnosed with TB (“symptomatic controls” - see definitions below). Children with DR-TB were also evaluated at 2 months by the study team. Further follow-up for children with DR-TB was completed by dedicated DR-TB services for children in Cape Town. Response to treatment at the 2 month visit was selected *a priori* as the primary time point for evaluating treatment response or clinical progress

given the turnaround time for mycobacterial culture, and the rationale for evaluation at the end of the intensive phase of antituberculosis treatment. In addition, the current NIH consensus criteria also recommend the 2 month time point for evaluation of treatment response or clinical progress<sup>174</sup>. For children who missed the 2 month visit, month 1 data was used if available: any child who had a month 2 visit, or who had month 1 follow-up but was not seen at month 2, was classified as having had follow-up.

At the 2 month follow-up visit, all children, regardless of TB diagnosis or TB (or other) treatment, underwent standard clinical evaluation, including:

- a) Assessment of clinical signs and symptoms compared to initial presentation,
- b) Anthropometric measurements,
- c) Evaluation of new TB exposure,
- d) Documentation of intercurrent illness or hospitalization,
- e) Chest radiography

All of these measures were completed in the same way as at baseline. Follow-up CXRs were dual read in the same manner, independently of the baseline film. Once evaluated, the follow-up film was compared to the previous film and changes recorded as “resolved”, “improved”, “unchanged” or “worse/different pathology”. If either the baseline or the follow-up films were technically unacceptable or not done or missing at the time of independent evaluation by expert readers, a comparison could not be made.

In addition to the above measures, in children diagnosed with TB (clinically or microbiologically confirmed), follow-up microbiological evaluation and adherence assessment were completed as per the schedule of evaluations (See Table 3.2).

**Microbiological evaluation at follow-up** was completed on two respiratory specimens of different types (typically one each of GA/ ESP depending on the child's age, and an IS), collected on the day of the visit, following the same SOP as per baseline (including observation of minimum fasting time). Smear microscopy, Xpert MTB/RIF, MGIT culture and DST were completed on all specimens as described above.

**TB case classification:** For the diagnostic sub-studies (Chapters 6 to 9) participants' TB disease status was classified using the revised NIH clinical case definitions for diagnostic research in paediatric PTB <sup>174</sup>, into the categories "Confirmed TB" (*M.tb* confirmed by culture or Xpert on at least one respiratory specimen or stool), "Unconfirmed TB" (no bacteriological confirmation of *M.tb* and a minimum two of well-defined TB symptoms; proof of TB infection or TB exposure in the past 12 months; CXR suggestive of TB; and favourable response to antituberculosis treatment at 2 months) and "Unlikely TB" (no bacteriological confirmation of *M.tb* and the criteria for "unconfirmed TB" not met). The case classification status was determined at the 2-month follow-up, when final culture results from enrolment were available and when response to treatment was evaluated.

Clinical groups by antituberculosis treatment status in the study:

- TB case: a child with clinically diagnosed or microbiologically confirmed TB.

- Symptomatic control: a child who, after baseline investigations, was assessed as not having active TB, and who was not initiated on antituberculosis treatment.
- Misclassified case: a child who was initially classified as a symptomatic control, but was diagnosed with TB before the month 2 visit (either because initial microbiology investigations returned positive results or because of lack of clinical improvement). The month 2 follow-up visit in these children was completed 2 months after antituberculosis treatment initiation, not 2 months after enrolment.

#### **Attrition to study**

- Lost to follow-up: a child whom the study team was not able to trace after a minimum of 3 varied attempts over a period of 3 months, including phone calls, and visits to the child's home or relevant health care clinics. The lost to follow-up date was the last date on which the child was seen (and data censored).
- Withdrawn: a child for whom the caregiver/legal guardian decided to withdraw from the study, or whom the study principal investigator withdrew if in the best interest of the child. The withdrawn date was the date on which the decision to withdraw was made (and data censored).

#### **Biological sampling for storage and future use**

All children had 2 mL serum and 5 mL urine collected at baseline for storage into a bio-repository. Any stool specimens remaining after all study testing had been completed was also stored. For children diagnosed with TB, follow-up serum and urine were also collected at month 2. These specimens will be used to evaluate novel

TB diagnostic and prognostic biomarkers for use in children in future (beyond the scope of this doctoral dissertation).

### **Ethics and regulatory approvals**

The study which supported this doctoral work was approved by the Stellenbosch University Health Research Ethics Committee in October 2011 (N11/09/282). Approval was renewed annually based on progress reports. The most recent approval is valid until February 2019, including for the doctoral analyses (See Appendix E). The Stellenbosch University Health Research Ethics Committee also gave approval for the collection and storage of specimens for bio-banking, for future use in the development and validation of novel diagnostic and prognostic markers for paediatric PTB. The study was approved by the relevant local health authorities governing the two participating hospitals.

### **Statistical analysis**

Clinical and demographic characteristics were summarized by clinical groups of interest using means and standard deviations if data were normally distributed, and with medians and inter-quartile ranges if non-normally distributed. STARD guidelines were followed for reporting and analyses<sup>189</sup>. Depending on the number of groups compared, the Wilcoxon ranksum or Kruskal-Wallis test were applied to compare medians, and the t-test or ANOVA for comparing means. Proportions were compared using the Chi squared or Fisher's exact tests, as appropriate.

Diagnostic analyses for sensitivity, specificity, positive and negative predictive values, and diagnostic yield, as well as logistic regression to evaluate factors associated with relevant diagnostic outcomes of interest, are described in detail in the

chapters focusing on specific diagnostic questions and analyses conducted as part of this doctoral research (See Chapters 6-9).



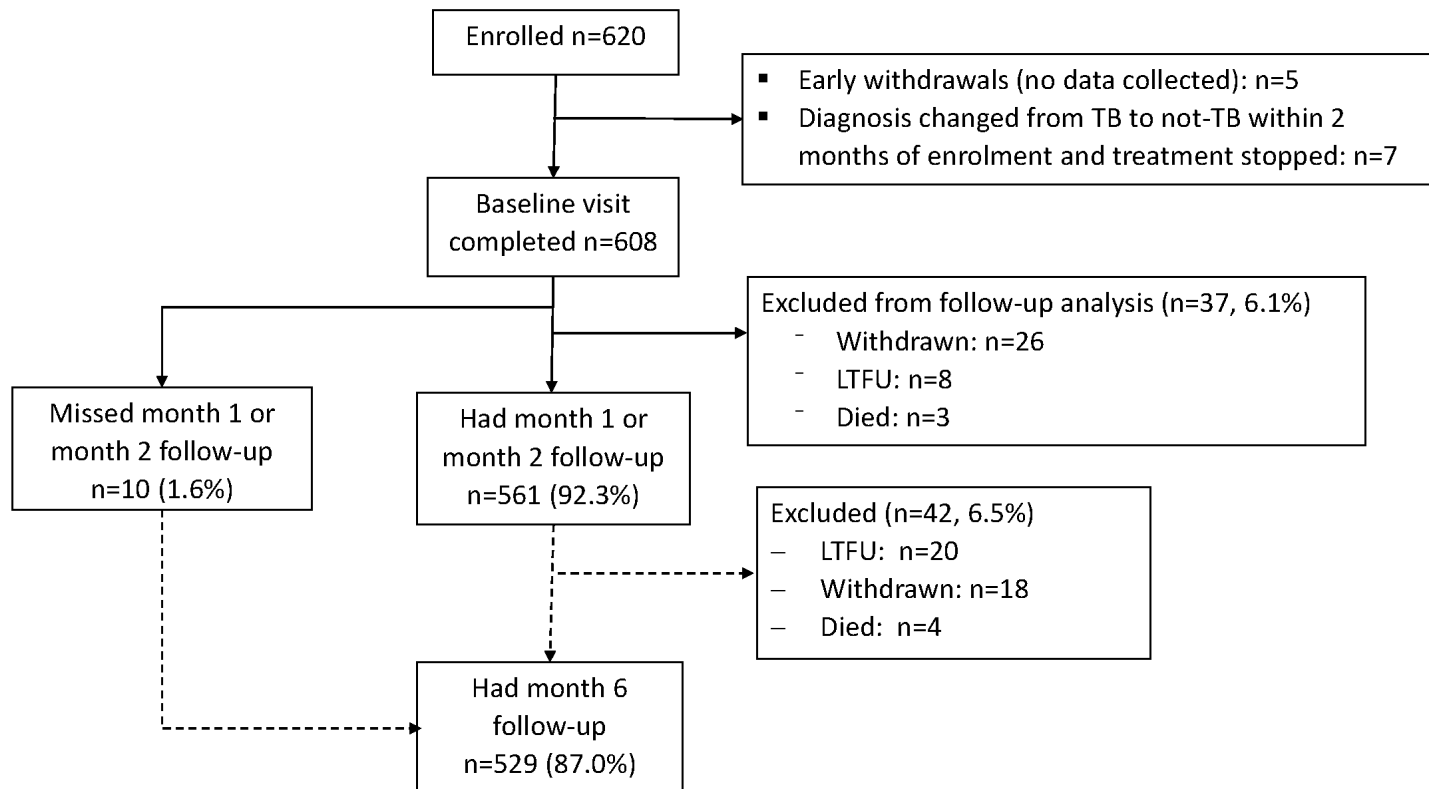
## **Chapter 4**

### **Clinical description of cohort**

#### **4.1 Enrolment and follow-up**

Over the study period, 620 children were enrolled, of whom 12 were early exclusions (Figure 4.1). Of the 608 children with a baseline visit, 561 (92.3%) were followed: 532 (87.5%) to month 2, and 29 (4.8%) who did not have month 2 visits completed, were followed at month 1 and were included in analysis (Figure 4.1). Three early deaths occurred, all in young children (aged between 8 and 17 months), all within 5 days of enrolment. One had severe adenoviral pneumonia which lead to respiratory failure. Two died of sepsis complicating severe acute malnutrition, gastroenteritis and shock. One of these children was HIV-infected, and not on ART.

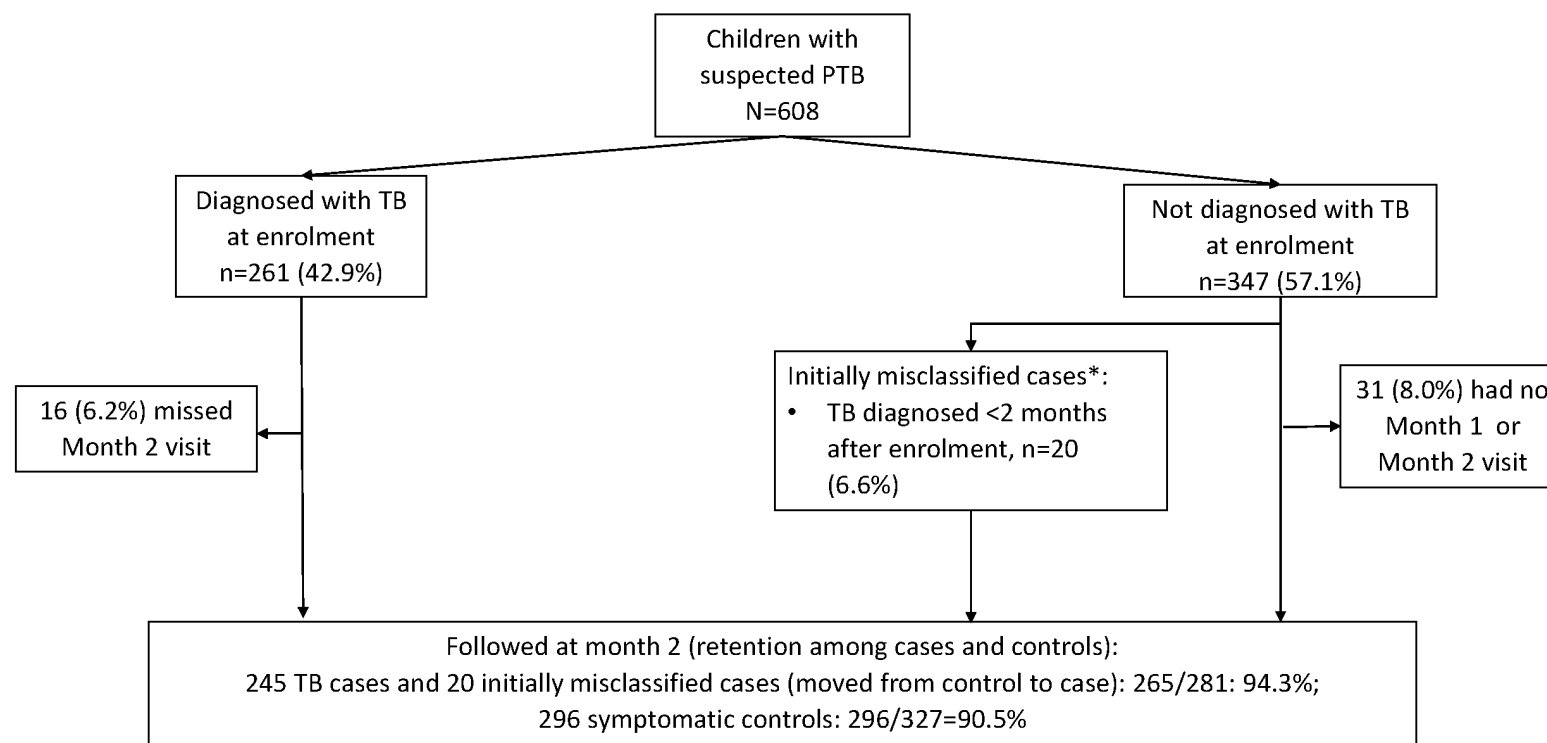
Ultimately, 529/608 (87.0%) children were followed to 6 months (Figure 4.1). Analysis for this doctoral dissertation is restricted to response to treatment and outcomes at 2 months.



*LTFU: Lost to follow-up*

**Figure 4.1. Overview of cohort flow for children enrolled in the study with suspected pulmonary (intrathoracic) tuberculosis and follow-up completed**

Among the children who were followed, 265/561 (47.2%) were TB cases (20 cases initially misclassified as not having TB), and 296/561 (52.8%) were symptomatic controls (Figure 4.2).



PTB: pulmonary (intrathoracic) tuberculosis

\*Baseline evaluation for misclassified cases was as per the initial presentation.

Follow-up evaluation was completed at 1 and 2 months - after TB treatment initiation (TB cases) and in symptomatic controls, regardless of treatment

**Figure 4.2. Follow-up of children with suspected pulmonary (intrathoracic) tuberculosis by TB disease status**

This chapter describes the overall clinical characteristics of the cohort. Chapter 5 describes the bacteriological findings in relation to specific clinical groups of interest, and detailed microbiological data.

## **4.2 Overview of cohort characteristics including clinical presentation at enrolment**

Overall, the cohort was young, with a median age of 16.7 months (inter-quartile range-IQR: 9.5-33.3), with only 10% of children being older than 5 years of age. There was a large proportion of infants, with 218 (35.9%) of children being younger than 12 months of age. Ninety (14.8%) children were below 6 months of age (Table 4.1).

Overall, 474 (77.8%) children had at least one of the well-defined symptoms of PTB described in the literature <sup>190</sup>, specifically prolonged unremitting cough<sup>1</sup> in 200 (32.9%), and lethargy/lack of playfulness in 244 (40.1%). The median cough duration in children who reported having any cough was 14 (IQR 5-21) days, and prolonged cough for  $\geq 14$  days was reported in 265 (43.6%). Failure to thrive occurred in 256 (42.1%) of cases of which 231/256 (90.2%) were observed from the child's growth chart, with 43 (18.6%) having evidence of weight loss over the preceding 3 months. In 25 children, the growth card was not available but the WFAZ was below -2 at enrolment. In 135/608 (22.2%) children, the clinical presentation lacked the typical symptoms of TB. TST positivity was documented in 128/460 (27.8%) and close TB exposure in 223/608 (36.8%).

Chest radiography was classified as being typical of TB in 181/565 (32.0%) evaluable films, while in 312 (55.2%), non-typical manifestations were reported, and in

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<sup>1</sup> See definitions, Chapter 3, page 45.

71 (12.6%), the films were reported as normal (Table 4.2). The majority (135/181; 74.6%) of films with typical features of TB showed intrathoracic lymph nodes, complicated in 34.1%, mostly resulting in large airway obstruction (28%). Over 10% of typical films had cavities, in the form of cavitating Ghon foci or TB bronchopneumonia in younger children and infants, or adult-type disease in older children. Radiologically severe disease was reported in almost half (49.2%) of children with CXRs classified as being typical of TB. EPTB together with PTB was diagnosed in 42 (6.9%) children, with some children having involvement of more than one extrapulmonary site. Abdominal TB was diagnosed in 16 (38.1%); miliary TB in 15 (35.7%), peripheral TB lymphadenitis in 12 (28.6%) and neurotuberculosis in 6 (14.3%).

Overall, 51 (8.4%) children had a history of prior antituberculosis treatment. Children with previous TB were older (median age 46.3 months) than those with no previous TB episodes (median age 15.5 months;  $p < 0.001$ ), and 24/51 (47.1%) were HIV-infected.

Antituberculosis treatment was initiated in 281/608 (46.2%) children within 2 months of enrolment, including in the 20 children who were initially misclassified as symptomatic controls.

Children who were followed up were similar to those who were not, for all demographic and clinical characteristics, except for the prevalence of prolonged cough, which was more common in the group who were followed up ( $p = 0.02$ ; Table 4.3).

The diagnoses in the children in the symptomatic control group were categorised into several broad groups: 25% had pneumonia or a lower respiratory tract infection; 21% had unspecified cough, with or without TB exposure/ failure to thrive; 18% had recurrent wheeze/ reactive airway disease associated with viral illness or atopy; 9% had failure to thrive with or without TB exposure; 5% had recurrent lower respiratory tract

infections including chronic protracted bronchitis; 5% had chronic lung disease, including HIV-associated, post-adenovirus bronchiolitis *obliterans* and prematurity-associated; 5% were HIV-infected children, either newly diagnosed or who had defaulted ART, and were symptomatic with abnormal CXR. The remaining 12% had a variety of conditions, including cardiac disease, genetic conditions (e.g. primary ciliary dyskinesia), pulmonary hydatid cysts and gastro-oesophageal reflux disease. In 10% of all symptomatic controls reviewed, one or more viruses were detected from blood (for cytomegalovirus) or respiratory secretions (data not shown).

### **4.3 Groups of specific interest**

#### **4.3.1 Characteristics in young infants vs. older children**

The nutritional status, HIV exposure and HIV infection status were similar in infants <6 months of age to infants >6 months and older children. A significantly lower proportion of infants, however, presented with well-defined TB symptoms (66.7% vs. 79.7%;  $p=0.003$ ), while the prevalence of TB exposure history, chest radiographic features typical of TB and microbiologically confirmed TB was similar to older infants and children (Table 4.4). The lower prevalence of well-defined symptoms in infants <6 months of age was due to a significantly lower proportion presenting with poor feeding and poor growth over the preceding 3 months, while the prevalence of persistent cough, wheezing and lethargy were similar to older infants and children. Of the 26 CXRs in infants <6 months of age showing features typical of TB, 22 (84.6%) had lymph node disease, which was complicated in 16 (72.7%). Six (23.1%) infants had cavitating disease, and four (15.4%) had a miliary pattern. Overall in this young group, radiologically severe TB was found in 21/26 (80.8%; data not shown). Six (6.9%) infants in this group had

EPTB with PTB: all six had miliary/disseminated TB. Three infants (50%) with miliary TB had no evidence of BCG vaccination<sup>2</sup>.

### 4.3.2 Characteristics in HIV-infected children

The overall HIV prevalence in the cohort was 11.8%. Only 32/72 (44.4%) HIV-infected children were receiving ART at enrolment, despite HIV-infected children being older (median age 21.9 months;  $p < 0.001$ ) than the rest of the cohort (Table 4.5). Twenty-seven (84.4%) of the 32 children were on ART for  $\geq 3$  months. Of the 5 children who had initiated ART within the 3 months prior to enrolment, only one was diagnosed with TB and may therefore have represented a case of TB immune reconstitution. Only one of the 72 HIV-infected children, in the symptomatic control group, was virally suppressed. HIV-infected children were also significantly more malnourished (median WFAZ -2.1 for HIV-infected vs. -1.6 for HIV-uninfected children;  $p = 0.003$ ) and stunted (median height/length-for-age Z-score -2.5 for HIV-infected vs. -1.7 for HIV-uninfected children;  $p = 0.001$ ). A significantly greater proportion of HIV-infected children had a history of previous antituberculosis treatment (24/72; 33.3%) compared to HIV-uninfected children (27/536; 5.0%,  $p < 0.001$ ). The prevalence of well-defined symptoms associated with paediatric PTB was similar in HIV-infected and uninfected children, as was the prevalence of TST positivity (27.8% overall), well-defined TB exposure (36.7% overall) and the proportion of microbiologically confirmed TB cases (23.6% among HIV-infected and 20.7% among HIV-uninfected children;  $p = 0.57$ ). Although overall, the proportion of children with a CXR typical of TB (32.4% overall) did not differ by HIV status, miliary disease was more prevalent in HIV-infected (5/24; 20.8%) compared to uninfected children (6/157; 3.8%,  $p < 0.001$ ). A significantly higher proportion of HIV-infected

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<sup>2</sup> See definition of BCG evidence, Chapter 3, page 48.

(12/72: 16.7%) vs. uninfected children (30/536; 5.6%;  $p=0.002$ ) had EPTB together with PTB. In four (33.3%) children, more than one extrapulmonary site was involved. Five (41.7%) had miliary disease and eight (66.7%) had abdominal TB. Overall, significantly more HIV-infected than uninfected children were initiated on antituberculosis treatment (58.3% vs. 40.7%;  $p=0.004$ ).

#### **4.3.3 TB cases initially misclassified as symptomatic controls**

There were 20 children (3.3% of the entire cohort; 6.6% of children initially classified as symptomatic controls) in this group (Figure 4.2): 9 had microbiologically confirmed TB, all by culture: 8 from baseline specimens (including one child confirmed only on stool culture) and one from a FNAB of a submandibular lymph node collected at the month 1 visit. This latter child had initially been treated for bacterial adenitis. Three children had multiple positive cultures. The remaining five had a single positive culture each: one from stool, the rest from respiratory specimens. Four children had maternal TB exposure (two were HIV-infected and one was an infant whose 6 year old sibling also had TB). The median TTP for positive *M.tb* cultures was 23 days (range 11 to 29 days), and the median time to antituberculosis treatment initiation from enrolment was 41 days (range 20 to 59 days). The median age of children in the misclassified group was 19.3 months (IQR: 14.8-46.9 months). Well-defined symptoms of TB were reported in 15 (75%), while 11 (55.0%) had close TB exposure and 3 (15%) were TST-positive. Only one of the misclassified cases with confirmed TB had a CXR that was assessed as typical of TB, while six had non-typical manifestations and 2 were assessed as normal at baseline. Of the remaining 11 children in whom TB was not confirmed, antituberculosis treatment was initiated at follow-up due to persistent or worsening symptoms: chest radiology was typical of TB in one, normal in one and not typical in seven, while in two the quality of the



films was not acceptable. Four (20%) children were initially prescribed preventive therapy before the diagnosis of TB was made and antituberculosis treatment was started.

**Table 4.1. Characteristics of all children enrolled with suspected pulmonary (intrathoracic) tuberculosis**

	<b>All (n=608)</b>
Median age in months (IQR)	16.7 (9.5-33.3)
Age group:	
– <6m	90 (14.8)
– 7-12m	128 (21.1)
– 13-24m	172 (28.3)
– 25-36m	80 (13.2)
– 37-60m	78 (12.8)
– >60m	60 (9.9)
Male (%)	324 (53.3)
BCG- vaccinated	590 (97.0)
HIV-exposed (%)	160 (26.3)
HIV-exposed uninfected (%)	99 (16.3)
HIV- infected* (%)	72 (11.8)
On ART (%)	32 (44.4)
Median time on ART in months (IQR)	17.8 (5.0-43.7)
On ART ≥3 months	27 (37.5)
Median CD4% (IQR)	21.7 (13.2-28.3) [n=65]
Median CD4 absolute, cells/μL (IQR)	912 (418-1261) [n=64]
Severity of immune suppression [n=65] <sup>191</sup>	
– Not significant	26 (40)
– Mild	9 (13.8)
– Advanced	8 (12.3)
– Severe	22 (33.8)
Median VL count, copies/mL (IQR)	142 812 (22 983- 893 935) [n=54]
Median VL log copies/mL (IQR)	5.2 (4.3-6.0) [n=54]
Median WFAZ (IQR)	-1.7 (-2.7 to -0.7)
Median HFAZ (IQR)	-1.8 (-2.9 to -0.6) [n=589]
Previous TB treatment** (%):	51 (8.4):
– <25m of age	- 14 (27.5)
– <61m of age	- 35 (68.6)
– HIV- infected	- 24 (47.1)
Well-defined TB symptoms (%)	474 (78.0)
– Prolonged unremitting cough ≥14 days	200 (32.9)
– Prolonged cough ≥ 14 days	265 (43.6)
– Prolonged wheeze [n=352]	45 (12.8)
– Lethargy/lack of playfulness	244 (40.1)
– Prolonged poor feeding/ poor appetite	113 (18.6)
– Failure to thrive	256 (42.1)
○ Crossing growth centiles/static growth > 3m	188 (30.9)

○ Weight loss > 3m	43 (7.1)
○ WFAZ <-2 with no previous measurements available	25 (4.1)
TST positive (%)	128 (27.9) [n=459]
Close TB exposure (%)	223 (36.7)
CXR typical of TB (%)	181 (32.1) [n=564]
TB microbiologically confirmed (%)	117 (19.4)
EPTB	42 (6.9)
TB disease status <sup>§</sup>	
– TB case	261 (42.9)
– Symptomatic control	327 (53.8)
– Initially misclassified case	20 (3.3)

*\*Not all HIV-infected children had documented perinatal exposure: 11 HIV-infected children had no documented or known HIV exposure are not included in the “HIV-exposed” group; \*\*No infant <12 months had previous TB; § See definitions in Chapter 3.*

*LTFU: lost to follow-up; IQR: inter-quartile range; m: months; BCG: bacille Calmette Guérin; ART: antiretroviral therapy; VL: viral load; WFAZ: weight-for-age Z-score; HFAZ: height/length-for-age Z-score; TST: tuberculin skin test (Mantoux); CXR: chest X-ray; EPTB: extrapulmonary (extrathoracic) tuberculosis.*

**Table 4.2. Chest radiographic findings of children enrolled with suspected pulmonary (intrathoracic) tuberculosis**

	<b>All, n=608 (100%)</b>
Normal	71 (11.7)
Typical of TB:	181 (29.8)
– Intrathoracic lymph node disease	135 (74.6)
○ Uncomplicated	89 (65.9)
○ Complicated	46 (34.1)
▪ Large airway obstruction	38 (28.1)
– Cavitating disease <sup>a</sup>	20 (11.0)
– Expansile pneumonia	10 (5.5)
– Ghon focus	4 (2.2)
– Miliary	11 (6.1)
– Pleural effusion	22 (12.2)
– Typical feature together with:	
○ Alveolar consolidation	91 (50.3)
○ Lobar consolidation	3 (1.7)
○ Interstitial infiltrates	2 (1.1)
○ Volume loss/collapse	1 (0.6)
– Extensive <sup>b</sup>	38 (21.0)
– Complicated	66 (36.5)
– Severe <sup>c</sup>	89 (49.2)
Not typical of TB:	312 (51.3)
– Alveolar consolidation (non-lobar)	182 (58.3)
– Interstitial infiltrates	64 (20.5)
– Perihilar infiltrates	55 (17.6)
– Lobar pneumonia	33 (10.6)
– Peripheral airway obstruction	15 (4.8)
– Volume loss/ Collapse	11 (3.5)
– Bronchiectasis	5 (1.6)
– Extensive <sup>b</sup>	36 (11.5)
– Complicated <sup>d</sup>	4 (1.3)
Unacceptable quality	36 (5.9)
Unavailable for dual reading	8 (1.3)

<sup>a</sup>Includes adult-type disease, cavitating Ghon focus and TB bronchopneumonia; <sup>b</sup> Extensive was defined as bilateral alveolar consolidation, or consolidation involving >2 zones, or consolidation approximating surface area greater than that of the right upper lobe; <sup>c</sup> Severity was defined as extensive or complicated pathology or miliary pattern (see Chapter 3 Methods); <sup>d</sup> The only features categorized as “complicated” for non-typical films were non-TB cavities.

**Table 4.3. Characteristics of all children with suspected pulmonary (intrathoracic) tuberculosis, comparing those who completed follow-up at month 2 to those who did not**

	All (n=608)	Followed (n=561)	Withdrawn/LTFU/ Died/ Missed month 1 and 2 follow up (n=47)
Median age in months (IQR)	16.7 (9.5-33.3)	16.7(9.5-33.3)	16.7 (7.1-28.8)
Age group:			
– <6m	90 (14.8)	82 (14.6)	8 (16.7)
– 7-12m	128 (21.1)	118 (21.0)	10 (20.8)
– 13-24m	172 (28.3)	158 (28.2)	14 (29.8)
– 25-36m	80 (13.2)	76 (13.5)	4 (8.3)
– 37-60m	78 (12.8)	70 (12.5)	8 (16.7)
– >60m	60 (9.9)	57 (10.2)	3 (6.3)
Male (%)	324 (53.3)	302 (53.8)	22 (46.8)
BCG-vaccinated	590 (97.0)	545 (97.1)	45 (95.7)
HIV -exposed (%)	160 (26.3)	146 (26.0)	14 (29.8)
HIV- exposed uninfected (%)	99 (16.3)	88 (15.7)	11 (23.4)
HIV infected (%)	72 (11.8)	69 (12.3)	3 (6.4)
On ART (%)	32 (44.4)	32 (46.4)	0 (0.0)
Median time on ART in months (IQR)	17.8 (5.0-43.7)	17.8 (5.0-43.7)	-
Median CD4 % (IQR)	21.7 (13.2-28.3) [n=65]	22.3 (13.2-28.3) [n=62]	18.9 (6.9-26.7) [n=3]
Median CD4 absolute, cells/ $\mu$ L (IQR)	912 (418-1261) [n=64]	922 (430-1258) [n=61]	725 (143-2049) [n=3]
Median VL count, copies/mL (IQR)	142 812 (22 983- 893 935) [n=54]	141 975 (15 585- 893 935) [n=55]	818842 (7 979 – 1 629 705) [n=2]
Median VL log copies/mL (IQR)	5.2 (4.3-6.0) [n=54]	5.2 (4.3-6.0) [n=55]	5.1 (3.9-6.2) [n=2]
Median WFAZ (IQR)	-1.7 (-2.7 to -0.7)	-1.7 (-2.8 to -0.7)	-1.7 (-2.6 to -1.0)
Median HFAZ (IQR)	-1.8 (-2.9 to -0.6) [n=589]	-1.8 (-2.9 to -0.6) [n=552]	-1.4 (-2.7 to -0.9) [n=37]
Previous TB treatment (%):	51 (8.4):	48 (8.6):	3 (6.4):
– <25m of age	14 (27.5)	13 (27.1)	1 (33.3)
– <61m of age	35 (68.6)	33 (68.8)	2 (66.7)
– HIV- infected	24 (47.1)	23 (47.9)	1 (33.3)
Well-defined TB symptoms (%)	474 (78.0)	440 (78.4)	34 (72.3)
– Prolonged unremitting cough $\geq$ 14 days	200 (32.9)	191 (34.0)	9 (19.1)
– Prolonged cough $\geq$ 14 days	265 (43.6)	252 (44.9)*	13 (27.7)*
– Prolonged wheeze	45 (12.8)	45 (13.6)	0

	[n=352]	[n=332]	[n=20]
– Lethargy/lack of playfulness	244 (40.1)	222 (39.6)	22 (46.8)
– Prolonged poor feeding/ poor appetite	113 (18.6)	109 (19.4)	4 (8.5)
– Failure to thrive	256 (42.1)	238 (42.4)	18 (38.3)
○ Crossing growth centiles/static growth > 3 m	188 (30.9)	176 (31.4)	12 (25.5)
○ Weight loss > 3 m	43 (7.6)	39 (7.0)	4 (8.5)
○ WFAZ <-2 with no previous measurements available	25 (4.1)	23 (4.1)	2 (4.3)
TST positive (%)	128 (27.9) [n=459]	118 (27.8) [n=425]	10 (29.4) [n=34]
Close TB exposure (%)	223 (36.7)	204 (36.4)	19 (40.4)
CXR typical of TB (%)	181 (32.0) [n=564]	170 (32.7) [n=520]	11 (25.0) [n=44]
TB microbiologically confirmed (%)	117 (19.4)	111 (19.8)	6 (12.8)
EPTB	42 (6.9)	38 (6.8)	4 (8.5)
TB disease status <sup>§</sup>			
– TB case	261 (42.9)	245 (43.7)	16 (34.0)
– Symptomatic control	327 (53.8)	296 (52.8)	31 (66.0)
– Initially misclassified case	20 (3.3)	20 (3.5)	0

\* This was the only statistically significant comparison;  $p=0.02$ ; § See definitions in Chapter 3. LTFU: lost to follow-up; IQR: inter-quartile range; m: months; BCG: bacille Calmette Guérin; ART: antiretroviral therapy; VL: viral load; WFAZ: weight-for-age Z-score; HFAZ: height/length-for-age Z-score; TST: tuberculin skin test (Mantoux); CXR: chest X-ray. EPTB: extrapulmonary (extrathoracic) tuberculosis. None of the comparisons were statistically significant.

**Table 4.4. Characteristics of all children with suspected pulmonary (intrathoracic) tuberculosis, comparing infants under 6 months of age to older infants and children**

	All (n=608)	Infants <6 months (n=90)	Infants and children >6 months (n=518)	p-value if significant
Male (%)	324 (53.3)	45 (50.0)	279 (53.9)	
BCG-vaccinated	590 (97.0)	85 (94.4)	505 (97.5)	
HIV- exposed (%)	160 (26.3)	27 (30.0)	133 (25.7)	
HIV- exposed uninfected (%)	99 (16.3)	20 (22.2)	79 (15.0)	
HIV- infected (%)	72 (11.8)	8 (8.9)	64 (12.4)	
On ART (%)	32 (44.4)	2 (25.0)	30 (46.2)	
Median time on ART in m (IQR)	17.8 (5.0-43.7)	2.8 (2.1-3.5)	21.6 (6.3-49.5)	Not calculated
Median CD4 % (IQR)	21.7 (13.2-28.3) [n=65]	22.0 (12.0-25.6) [n=6]	21.7 (13.2-29.0) [n=59]	
Median CD4 absolute, cells/ $\mu$ L (IQR)	912 (418-1261) [n=64]	980 (838-1487) [n=6]	911.5 (405-1258) [n=58]	
Median VL count, copies/mL (IQR)	142 812 (22 983- 893 935) [n=56]	762 440 (36 830- 1 633 157) [n=6]	135 062 (16665 - 893 935) [n=50]	
Median VL log copies/mL (IQR)	5.2 (4.3-6.0) [n=56]	5.8 (4.6-6.2) [n=6]	5.1 (4.2-6.0) [n=50]	
Median WFAZ (IQR)	-1.7 (-2.7 to - 0.7)	-1.9 (-2.9 to - 0.7)	-1.6 (-2.7 to - 0.7)	
Median HFAZ (IQR)	-1.8 (-2.9 to -0.6) [n=589]	-2.0 (-3.6 to -0.9) [n=88]	-1.7 (-2.8 to -0.6) [n=501]	
Well-defined TB symptoms (%)	474 (78.0)	60 (66.7)	414 (79.9)	0.003
– Prolonged unremitting cough $\geq$ 14 days	200 (32.9)	27 (30.0)	173 (33.4)	
– Prolonged cough $\geq$ 14 days	265 (43.6)	33 (36.7)	232 (44.8)	
– Prolonged wheeze	45 (12.8) [n=352]	5 (12.5) [n=40]	40 (12.8) [n=312]	
– Lethargy/lack of playfulness	244 (40.1)	36 (40.0)	208 (40.2)	
– Prolonged poor feeding/ poor appetite	113 (18.6)	2 (2.2)	111 (21.4)	<0.001
– Failure to thrive	256 (42.1)	17 (18.9)	239 (46.1)	<0.001
○ Crossing growth centiles/static growth > 3 m	188 (30.9)	11 (12.2)	177 (34.2)	<0.001

○ Weight loss > 3m	43 (7.1)	1 (1.1)	42 (8.1)	0.02
○ WFAZ <-2 with no previous measurements available	25 (4.1)	5 (5.5)	20 (3.9)	0.48
TST positive (%)	128 (27.9) [n=459]	18 (27.7) [n=65]	110 (27.9) [n=394]	
Close TB exposure (%)	223 (36.7)	40 (44.4)	183 (35.3)	
CXR typical of TB (%)	181 (32.1) [n=564]	26 (31.3) [n=83]	155 (32.2) [n=481]	
TB microbiologically confirmed (%)	117 (19.2)	19 (21.1)	108 (20.9)	
EPTB	42 (6.9)	6 (6.7)	36 (6.9)	
TB status				
– TB case	261 (42.9)	33 (36.7)	228 (44.0)	
– Symptomatic control	327 (53.8)	57 (63.3)	270 (52.1)	
– Misclassified case	20 (3.3)	0 (0)	20 (3.9)	

*IQR: inter-quartile range; BCG: bacille Calmette Guérin; ART: antiretroviral therapy; m: months; VL: viral load; WFAZ: weight-for-age Z-score; HFAZ: height/length-for-age Z-score; TST: tuberculin skin test (Mantoux); CXR: chest X-ray; EPTB: extrapulmonary (extrathoracic) tuberculosis.*



**Table 4.5. Characteristics of all children with suspected pulmonary (intrathoracic) tuberculosis, comparing HIV-infected to HIV-uninfected children**

	All (n=608)	HIV-infected (n=72)	HIV- uninfected (n=536)	p-value (significant values only)
Median age in months (IQR)	16.7 (9.5-33.1)	29.1 (13.8-59.4)	16.0 (9.3-30.0)	<0.001
Age group:				
– <6m	90 (14.8)	8 (11.1)	82 (15.3)	0.35
– 7-12m	128 (21.1)	9 (12.5)	119 (22.2)	0.06
– 13-24m	172 (28.3)	13 (18.1)	159 (29.7)	0.04
– 25-36m	80 (13.2)	11 (15.3)	69 (12.9)	0.57
– 37-60m	78 (12.8)	13 (18.1)	65 (12.1)	0.15
– >61m	60 (9.9)	18 (25.0)	42 (7.8)	<0.001
Male (%)	324 (53.3)	34 (47.2)	290 (54.1)	
BCG-vaccinated	590 (97.0)	71 (98.6)	519 (96.8)	
Median WFAZ (IQR)	-1.7 (-2.7 to -0.7)	-2.1 (-3.2 to -1.4)	-1.6 (-2.7 to -0.7)	0.003
Median HFAZ (IQR)	-1.8 (-2.9 to -0.6) [n=589]	-2.5 (-3.3 to -1.4) [n=70]	-1.7 (-2.9 to -0.6) [n=524]	0.001
Previous TB (%):	51 (8.4)	24 (33.3)	27 (5.0)	<0.001
Well-defined TB symptoms (%)	474 (78.0)	59 (81.9)	415 (77.4)	
– Prolonged unremitting cough ≥ 14 days	200 (32.9)	26 (36.1)	174 (32.5)	
– Prolonged cough ≥ 14 days	265 (43.6)	38 (52.8)	227 (42.4)	
– Prolonged wheeze [n=352]	45 (12.8) [n=352]	4 (9.8) [n=41]	41 (13.2) [n=311]	
– Lethargy/lack of playfulness	244 (40.1)	38 (52.8)	206 (38.4)	0.02
– Prolonged poor feeding/ poor appetite	113 (18.6)	7 (9.7)	106 (19.8)	0.04
– Failure to thrive	256 (42.1)	32 (44.4)	224 (41.8)	
○ Crossing growth centiles/static growth > 3 m	188 (30.9)	20 (27.8)	168 (31.3)	
○ Weight loss > 3 m	43 (7.1)	6 (8.3)	37 (6.9)	
○ WFAZ < -2 with no previous measurements available	25 (4.1)	6 (8.3)	19 (3.5)	0.05
TST positive (%)	128 (27.9) [n=459]	13 (26.5) [n=49]	115 (28.0) [n=410]	
Close TB exposure (%)	223 (36.7)	24 (33.3)	199 (37.1)	
CXR typical of TB (%)	181 (32.4) [n=565]	24 (34.8) [n=69]	157 (31.7) [n=496]	
TB microbiologically confirmed (%)	117 (19.2)	17 (23.6)	110 (20.5)	

EPTB	42 (6.9)	12 (16.7)	30 (5.6)	0.002
TB status				
– TB case	261 (42.9)	42 (58.3)	219 (40.9)	0.004
– Symptomatic control	327 (53.8)	27 (57.4)	300 (56.0)	
– Misclassified case	20 (3.3)	3 (6.4)	17 (3.2)	

*IQR: inter-quartile range; m: months; BCG: bacille Calmette Guérin WFAZ: weight-for-age Z-score; HFAZ: height/length-for-age Z-score; TST: tuberculin skin test (Mantoux); CXR: chest X-ray; EPTB: extrapulmonary (extrathoracic) tuberculosis.*

## **4.4 Discussion**

This well-defined prospective cohort is representative of young children with suspected PTB from high TB and HIV-burden settings, who are frequently referred to hospital for TB investigation and diagnosis. Children were mostly young, with a median age below 18 months, and a considerable proportion was below 6 months of age. There was high cohort retention at 2 months, with >90% of children having had a follow-up visit, despite the observational nature of this study.

### **4.4.1 Overall symptoms and spectrum of disease**

A high proportion of children presented with the well-defined symptoms classically associated with PTB in the literature <sup>6,190</sup>, reflecting the study's eligibility criteria. Notably, >20% of children did not have any of the prolonged, persistent symptoms characteristic of paediatric PTB. In these children, evidence of TB exposure, TB infection and suggestive chest radiology were the reasons for referral and routine TB investigation. Expert evaluation of chest radiology resulted in almost a third of films being classified as typical of paediatric PTB, while just over 50% were non-typical and 11% were assessed as being normal. The feature of typical TB disease most frequently reported, uncomplicated intrathoracic lymph node disease, reflects the most common manifestation of PTB in children, based on the natural history data of TB in children <sup>6</sup>. The radiological severity of TB disease observed in this cohort was representative of both severe and non-severe TB, which were equally represented. A relatively high proportion of cavitating disease (11%) was observed across all age groups, with an unexpectedly high proportion (23%) seen in typical films from infants <6 months of age. This has been previously described <sup>27,192</sup>, and highlights that

despite recent advances in the prevention<sup>193,194</sup> and rapid detection of TB<sup>25,195</sup> in adults and children, young infants experience rapid progression to severe disease after infection with *M.tb*. Non-typical radiological patterns included a high proportion of alveolar and interstitial infiltrates, reflecting a wide spectrum of both infectious and non-infectious lower respiratory tract pathology in young children investigated for PTB in this setting. This was reflected in the alternative diagnoses, which included a large proportion of acute and recurrent lower respiratory tract infections and reactive airway disease due to atopy and viral infections, with viruses identified in 10% of children.

A high proportion of children had previously received antituberculosis treatment. Although children with previous TB were older than those with no prior history of TB, the median age was still young, below 4 years of age, and reflects the high burden of ongoing exposure to *M.tb*.

#### **4.4.2 Characteristics of HIV-infected children**

The prevalence of HIV exposure in the cohort mirrors the current antenatal prevalence among pregnant women in the Western Cape province, South Africa during the study period [18.9% (95% confidence interval-CI: 16.3% – 22.0%)], while the relatively low HIV prevalence in this cohort reflects the increasing success of the PMTCT program in this province. However, HIV-infected children in this cohort had a high burden of co-morbidities, and a low percentage (<50%) were accessing ART at the time of TB investigation. Only one of 30 (3.3%) HIV-infected children on ART had virological suppression at enrolment. This is particularly concerning given that the median age of HIV-infected children was higher than for HIV-uninfected children, and

considering that the Western Cape province had already adopted early HIV PCR testing at 6 weeks during 2010, and increasingly, HIV PCR testing at birth (since 2015) with immediate ART initiation for all infants testing HIV-positive. These data again highlight the importance of TB investigation as a gateway for HIV testing and treatment initiation, and ART adherence support, given how interlinked these two epidemics are in children in settings like South Africa.

As previously reported, HIV-infected children presented with similar respiratory symptoms as HIV-uninfected children <sup>68,81,196</sup>, but were more malnourished and stunted than other children in the cohort. Over a third of HIV-infected children had previously been diagnosed with TB, emphasizing the extreme burden of disease experienced by HIV-infected children living in high TB-burden settings, and probably a degree of over-diagnosis, which is an indication of the diagnostic difficulties facing healthcare workers treating HIV-infected children in these settings <sup>19,196</sup>. HIV-infected children diagnosed with TB at the current presentation and TB investigation had a high prevalence of disseminated TB disease, with >20% having miliary TB and 16.7% having involvement of extrapulmonary sites as well as pulmonary sites, which was significantly higher than in HIV-uninfected children. Although the proportion of HIV-infected with CXRs typical of TB was similar regardless of HIV infection status <sup>197</sup>, the spectrum of TB disease reflected significantly more miliary disease in HIV-infected children. There are inconsistencies in published data on the spectrum of TB disease observed in HIV-infected children, with some studies, especially in settings where ART was not initiated early, reporting a higher risk of extrapulmonary dissemination with HIV infection <sup>53,198</sup>, while others

have observed no difference between HIV-infected and uninfected children <sup>81,199</sup>. At TBH, an unexpected high incidence of EPTB in HIV-infected children <2 years of age after initiation of ART has previously been reported <sup>17</sup>. Explanations for these conflicting results may include the age and stage of HIV disease at presentation <sup>15</sup>, use of ART, access to TB diagnostic services, including specialist radiology, and differences in the interpretation of diagnostic tests and imaging by clinicians and researchers <sup>200</sup>. In the present cohort, the high prevalence of miliary and extrapulmonary TB combined with the relatively low prevalence of complicated intrathoracic TB and the poor viral control in HIV-infected children suggest poor immunological containment of *M.tb*: a vigorous immunological response is partly responsible for the more typical radiological manifestations of TB, including enlargement of mediastinal lymph nodes and adult-type disease.

While it is known that HIV-infected children bear a high burden of opportunistic infections, with TB the most common of these in high burden settings <sup>81,201,202</sup>, early HIV diagnosis and delivery of ART are effective in reducing the incidence of TB in HIV-infected children <sup>81,203-205</sup>. Missed opportunities for early HIV diagnosis and initiation of ART in hospitalised children have recently been reported in our setting within the context of routine TB investigation and diagnosis <sup>206</sup>, and are recognised as a persistent barrier against achieving the 90-90-90 targets set for 2020 by the Joint United Nations Programme on HIV/AIDS <sup>207</sup>. Reasons for poor access to HIV diagnosis and ART are multiple, and beyond the scope of this dissertation, but require urgent intervention in order to improve the health of children living with HIV, and to prevent morbidity and mortality caused by opportunistic infections such as TB

in HIV-infected children, in whom ART should be initiated early to prevent both death and TB <sup>208</sup>.

#### **4.4.3 Characteristics of infants younger than 6 months of age**

A second population of interest which was well-represented in this cohort, were very young infants below 6 months of age, reflecting the very high burden of respiratory illness observed from an early age in our study population <sup>70,209,210</sup>, and the high risk of exposure to *M.tb* early in life in this setting <sup>211</sup>. Caregivers of infants <6 months of age reported similar patterns of prolonged cough and wheeze in their infants as in older infants and children. Although a lower proportion of infants <6 months had well-defined symptoms, this was due to the lower prevalence of failure to thrive and of poor feeding in this very young group, rather than to a more acute presentation (e.g. shorter duration of symptoms). This suggests that acute presentation in PTB, including as acute severe pneumonia <sup>45</sup>, is associated with factors other than age. An acute presentation has been documented in up to 20% of studies reporting TB as a co-diagnosis in acute pneumonia in children <sup>45</sup>. This figure closely matches what was observed in this cohort, in which 22% of children overall, and 18% of those diagnosed with PTB (see Chapter 5) presented with none of the prolonged, well-defined symptoms of paediatric PTB. Diagnosis of PTB is especially challenging in this group of children. A better understanding of the factors associated with such acute presentations, including underlying immunology and co-infection with other pathogens, is beyond the scope of this dissertation but is identified as a critical aspect needing in-depth study in future. Future diagnostic strategies and tools for paediatric TB should consider clinical scenarios which are almost exclusively

observed in children, including the relevant age spectrum, in order to truly meet the needs of these vulnerable populations.

Similarly to HIV-infected children, a high proportion of CXRs typical of TB in infants <6 months (15%) showed miliary spread. In addition, compared to older infants and children, these young infants had a significantly higher prevalence of radiologically severe TB (>80%), manifesting primarily as complicated lymph node disease with large airway obstruction. Airway obstruction is a well-documented manifestation of PTB in young children, due to the small calibre of infant airways<sup>98</sup>. Although BCG coverage was high overall (97%), it is notable that three of the six infants with miliary TB had no evidence of BCG vaccination, emphasizing the importance of ensuring that all infants from high TB burden settings have access to BCG to prevent the potentially catastrophic sequelae of disseminated TB. During the study period, there was a temporary global and also South African stock-out of BCG (occurring from 2015), which may have contributed the non-vaccination of these infants.

In summary, I demonstrate that although well-defined symptoms of PTB were reported in the majority of children in this study, as expected given the eligibility criteria, over 20% of children had a non-specific clinical presentation. Acute respiratory symptoms had similar prevalence across all age groups, and the acute presentation of PTB in children needs to be further explored. Radiological features not typically associated with PTB were common, and indicate a high burden of respiratory pathology as well as potentially non-typical radiological manifestations of PTB, which require further study. Young infants <6 months of age and HIV-infected



children were high-risk groups at risk of disseminated TB: targeted interventions for prevention, early screening and rapid diagnosis should consider the epidemiological, immunological and clinical determinants of the severe manifestations of TB observed in these vulnerable paediatric populations.

## **Chapter 5**

# **Mycobacteriological yield in children with suspected intrathoracic tuberculosis**

### **5.1 Overall characteristics of children by mycobacteriological confirmation and TB disease status**

Overall, after baseline evaluation, a diagnosis of PTB was made in 281/608 (46.2%) children. Of these, 117/281 (41.6%) had microbiologically confirmed TB, while 164 (58.4%) were clinically diagnosed (Table 5.1). Eight (6.8%) children in the confirmed group and 12 (7.3%) in the clinically diagnosed group were initially misclassified as being symptomatic controls at the baseline evaluation. In 327 children initially investigated for suspected PTB, an alternative diagnosis was made and antituberculosis treatment was not prescribed.

Children with microbiologically confirmed TB were significantly older (median age 21.1 months) than the clinically diagnosed (median age 18.4 months) group and symptomatic controls (median age 14.9 months;  $p=0.003$ ). Over 20% of children in the bacteriologically confirmed group were over 5 years of age, compared to approximately 7% for the other two groups. Conversely, a high proportion of children >5 years of age diagnosed with TB were microbiologically confirmed (24/37: 65.9%). Among infants <6 months of age diagnosed with TB, a high proportion, 18/33 (54.5%), were microbiologically confirmed.

The HIV prevalence among confirmed cases was similar to that in the overall cohort (11.1% among confirmed cases; 11.8% in the cohort), but a significantly higher prevalence of HIV infection was observed among clinically diagnosed cases (19.5%), together with a lower HIV prevalence in the symptomatic controls (8.3%;  $p=0.001$ ).

Nutritional status, perinatal HIV exposure and the prevalence of well-defined TB symptoms were similar across the three TB diagnostic groups.

Compared to the clinically diagnosed TB group, a significantly higher proportion of children with confirmed TB had a history of close TB exposure (50.4% vs 37.8%;  $p=0.001$ ), evidence of TB infection (69.9% vs. 40.8%;  $p<0.001$ ) and a CXR typical of TB (75.5% vs. 37.5%;  $p<0.001$ ). Among symptomatic controls, these proportions were lower (31.2%; 8.2% and 12.7%, respectively;  $p<0.001$ ). Among the 140 children with bacteriologically confirmed and clinically diagnosed PTB who had radiological features typical of PTB, 75 (53.6%) had severe PTB: *M.tb* was detected in 57/75 (76.0%) children with severe vs. 26/65 (40.0%) with non-severe PTB ( $p<0.001$ ).

Notably, 41 (12.7%) children in the symptomatic control group had CXRs reported as being “typical of TB”, including 28 with intrathoracic lymph nodes, 7 with pleural effusions, 5 children with cavities (2 also with lymph nodes), one with expansile pneumonia and one with a Ghon focus. In one case, readers agreed on the certainty of TB, but not on the radiological pattern. The clinical diagnoses among these 41 children with TB-compatible CXRs varied: 12 (29%) had one or more viruses isolated from the respiratory tract, including 4 with adenovirus; 7 (17%) had reactive airway disease/recurrent wheezing; 4 children had bronchiolitis *obliterans* (3 secondary to adenovirus); 3 had HIV-associated lung disease and 2 had non-TB necrotic pneumonia. The rest mostly had acute and recurrent lower respiratory tract infections with no identified aetiology. There was also one case of pulmonary hydatid cyst; one Langerhans

cell histiocytosis and one HIV-infected child with complex congenital cardiac disease and pulmonary hypertension.

At follow-up, 24/41 (58.5%) of this subgroup of children with CXRs compatible with PTB had symptomatic or other improvement (radiology improved in 15, in 2 remained abnormal and could not be assessed due to poor quality/non-availability in 7), while in 13 (31.7%) children, chest radiographic findings resolved or improved but symptoms persisted. One child, diagnosed with bronchiolitis *obliterans* secondary to severe adenoviral pneumonitis, had lack of resolution of both symptoms and chest radiographic findings at follow-up. Three children did not have follow-up evaluation. None of the 41 children received antituberculosis treatment.

## **5.2 Detailed bacteriological results for children with microbiologically confirmed TB**

*M.tb* was detected by culture or Xpert in 117/281 (41.6%) children classified as having TB. Overall, 94 (80.3%) had drug-susceptible TB: 84 (89.4%) were rifampicin and isoniazid-susceptible on LPA (81 respiratory specimens, 2 FNAB and one stool), and 10 with negative cultures were rifampicin-susceptible on Xpert (8 respiratory specimens, one FNAB and one stool). Four children with no DST results were treated as DS-TB (and had no known DR-TB exposure). Among the 19/117 (16.2%) children with first-line drug resistance on DST (either isoniazid or rifampicin resistance), 8 (42.1%) were rifampicin and isoniazid resistant on LPA (2 also resistant to amikacin and ofloxacin; one resistant to ofloxacin and susceptible to amikacin), 6 (31.6%) were isoniazid mono-resistant and 5 (26.3%) were rifampicin mono-resistant. All 5 of these isolates which were rifampicin-resistant and susceptible to isoniazid on LPA, were also rifampicin-resistant on Xpert. One child was assessed as having hetero-resistance: the child was 4 years old, HIV-

infected with severe immune suppression (CD4 1.3%, 21 cells/ $\mu$ L) and poor adherence to ART. The CXR at presentation showed cavitating disease. The child had completed a 6 month course of antituberculosis treatment for DS-TB three months prior to enrolment, during which adherence was uncertain. At enrolment, one GA was rifampicin-resistant on Xpert, but susceptible to both rifampicin and isoniazid on LPA. Two other respiratory specimens collected at enrolment were rifampicin-susceptible on both Xpert and LPA. Given the child's poor adherence history, genotyping was completed on the *M.tb* isolate from the GA with rifampicin resistance detected by Xpert. A 533 CTG-CCG mutation of the *rpoB* gene was identified, the *M.tb* strain belonging to the same family as the wild-type strain cultured from the first TB episode. The child was therefore treated with a DR-TB regimen.

Specific sub-groups of interest are discussed below (Table 5.2).

### 5.2.1 Xpert and culture-positive cases

Among the 117 microbiologically confirmed PTB cases, 61 (52.1%) had respiratory specimens which were both Xpert and culture positive. All smear-positive cases were in this group, with 21/61 (34.4%) children having at least one smear-positive respiratory specimen. All isoniazid mono-resistant cases (n=6) were also in this group, with 5 of the 21 (23.8%) smear-positive cases being isoniazid mono-resistant. Of 18 infants <6 months of age with microbiologically confirmed PTB, 16 (88.9%) were in this high-bacillary load category (Table 5.3), and 6 (33.3%) had smear-positive disease. These proportions were higher than for children >5 years of age, of whom 12/24 (50.0%) of confirmed cases were Xpert positive culture positive, and 6/24 (25.0%) were smear-positive. Three children had 3+ smear positive specimens: two were >5 years of age and had adult-type cavitating disease. The third was an infant <6 months old, with expansile

pneumonia and evidence of alveolar break-down: the lowest culture TTP in this infant was 3 days. All three of these cases had drug-susceptible TB.

### **5.2.2 Xpert-negative but culture positive cases**

In 40 (34.2%) children, all Xpert results were negative, but cultures from respiratory specimens were positive. Drug resistance was detected in 6/40 (15.0%): one (2.5%) had rifampicin mono-resistance; and 5 (12.5%) were resistant to both rifampicin and isoniazid, with one also resistant to amikacin and one to both amikacin and ofloxacin. The lowest median TTP for cultures from children in this category was significantly longer than that of children in the Xpert positive culture positive category (26 vs. 15 days;  $p < 0.001$ ).

### **5.2.3 Cases positive by Xpert only**

In 11/117 (9.4%) children classified as having TB, all respiratory cultures were negative, with *M.tb* detected only by Xpert. The lowest median Ct value in this group was significantly longer than in the Xpert positive culture positive group (31 vs. 26;  $p < 0.001$ ). In 8 (72.7%) cases, rifampicin resistance was not detected, while in 3 (27.3%), the rifampicin result was indeterminate due to probe delays (data not shown).

### **5.2.4 *M.tb* detected from only non-respiratory samples**

In five (4.3%) of 117 microbiologically confirmed cases with clinical evidence of PTB, test results on all respiratory specimens were negative, but *M.tb* was detected on FNAB in three (two by culture and one on Xpert, all drug-susceptible), and on stool in two (one by Xpert and one by culture, also drug-susceptible; also refer to Chapters 6 and 7).

### **5.2.5 Acid-fast bacilli detected from a single smear with no subsequent confirmation**

Three children who were smear positive, Xpert negative, and culture negative were not included in the n=117 cases, as they were not considered to have confirmed TB: each had a single GA which was scanty smear positive; these specimens were all collected at peripheral hospitals, not by the research team, and there was a concern regarding potential laboratory cross-contamination or environmental contamination with non-tuberculous mycobacteria. All subsequent samples collected by the research team were negative on all tests (smear, culture and Xpert). Two of these children were treated as TB cases by attending clinicians, while one was not treated and remained a “symptomatic control”.

**Table 5.1. Characteristics of children by TB diagnostic status, comparing children with confirmed TB, children with clinically diagnosed TB and symptomatic controls**

	All (n=608)	Confirmed TB (n=117)	Clinically diagnosed TB [n=164]	Symptomatic controls* [n=327]	p- value if signific ant
Age in months (IQR)	16.7 (9.5-33.1)	21.1 (10.4-53.4)	18.4 (10.7-31.4)	14.9 (8.2-28.6)	0.003
Age group:					
– < 6 m	90 (14.8)	18 (15.4)	15 (9.2)	57 (17.4)	
– 7-12 m	128 (21.1)	21 (18.0)	37 (22.6)	70 (21.4)	
– 13-24 m	172 (28.3)	24 (20.5)	51 (31.1)	97 (29.7)	
– 25-36 m	80 (13.2)	13 (11.1)	28 (17.1)	39 (11.9)	
– 37-60 m	78 (12.8)	17 (14.5)	20 (12.2)	41 (12.5)	
– > 61m	60 (9.9)	24 (20.5)	13 (7.9)	23 (7.0)	
Male (%)	324 (53.3)	51 (43.6)	93 (56.7)	180 (55.1)	0.06
HIV- exposed (%)	160 (26.3)	33 (28.2)	47 (28.7)	80 (24.5)	
HIV- exposed uninfected (%)	99 (16.3)	22 (18.8)	21 (12.8)	56 (17.1)	
HIV- infected** (%)	72 (11.8)	13 (11.1)	32 (19.5)	27 (8.3)	0.001
On ART (%)	32 (44.4)	8 (61.5)	13 (40.6)	11 (40.7)	
Median time on ART in m (IQR)	17.8 (5.0-43.7)	16.9 (8.2-59.3)	25.9 (6.6-37.8)	6.4 (2.1-33.9)	
Median CD4 % (IQR)	21.7 (13.2-28.3) [n=65]	26.9 (18.3-35.9) [n=11]	16.2 (12.1-26.7) [n=29]	22.4 (13.6-29.0) [n=24]	
Median CD4 cells/ $\mu$ L (IQR)	912 (418-1261) [n=64]	1176 (825-1605) [n=11]	839 (430-1258) [n=29]	876.5 (238-1278) [n=24]	
Median VL copies/mL (IQR)	142 812 (22 983- 893 935) [n=56]	81235 (44892- 226239) [n=9]	135063 (3089- 607510) [n=26]	204174 (16665- 893935) [n=21]	
Median VL log copies/mL (IQR)	5.2 (4.3-6.0) [n=56]	4.91 (4.7-5.4) [n=9]	5.1 (3.5-6.2) [n=26]	5.3 (4.2-6.0) [n=21]	
Median WFAZ (IQR)	-1.7 (-2.7 to -0.7)	-1.8 (-3.0 to -1.0)	-1.9 (-3.0 to -0.7)	-1.5 (-2.6 to -0.6)	
Median HFAZ (IQR)	-1.8 (-2.9 to -0.6) [n=589]	-2.0 (-3.0 to -0.9) [n=114]	-1.8 (-3.1 to -0.6) [n=161]	-1.7 (-2.8 to -0.6) [n=313]	
Well-defined TB symptoms (%)	474 (78.0)	95 (81.2)	133 (81.1)	246 (75.2)	
– Prolonged unremitting cough $\geq$ 14 days	200 (32.9)	48 (41.0)	56 (34.2)	96 (29.4)	



– Prolonged cough $\geq$ 14 days	265 (43.6)	62 (53.0)	71 (43.3)	132 (40.4)	
– Prolonged wheeze	45 (12.8) [n=352]	8 (11.8) [n=68]	13 (14.6) [n=89]	24 (12.3) [n=195]	
– Lethargy/lack of playfulness	244 (40.1)	52 (44.4)	71 (43.3)	121 (37.0)	
– Prolonged poor feeding/ poor appetite	113 (18.6)	27 (23.1)	36 (22.0)	50 (15.3)	
– Failure to thrive	256 (42.1)	53 (45.3)	77 (47.0)	126 (38.5)	
○ Crossing growth centiles/static growth >3m	188 (30.9)	31 (26.5)	58 (35.4)	99 (30.3)	
○ Weight loss >3m	43 (7.1)	13 (1.1)	14 (8.5)	16 (4.9)	
○ WFAZ <-2 with no previous measurements available	25 (4.1)	9 (7.7)	5 (3.0)	11 (3.4)	
TST positive (%)	128 (27.9) [n=459]	58 (69.9) [n=83]	49 (40.8) [n=120]	21 (8.2) [n=256]	<0.001
Close TB exposure (%)	223 (36.7)	59 (50.4)	62 (37.8)	102 (31.2)	0.001
CXR typical of TB (%)	181 (32.1) [n=564]	83 (75.5) [n=110]	57 (37.5) [n=152]	41 (13.6) [n=302]	<0.001
Radiologically severe PTB	89 (49.2) [n=181]	57 (68.7) [n=83]	18 (31.6) [n=57]	14 (34.1) [n=41]	<0.001
Radiologically extensive alveolar disease if CXR not typical of TB	38 (9.9) [n=383]	2 (7.4) [n=27]	11 (11.6) [n=95]	25 (9.6) [n=261]	
EPTB	42 (6.9)	27 (23.1)	15 (9.2)	0	
Antituberculosis treatment regimen <sup>§</sup> :					
– DS-TB	236 (38.8)	90 (76.9)	146 (89.0)	-	0.006
– DR-TB	45 (7.4)	27 (23.1)	18 (11.0)	-	

*\*One child presented with wheezing, and upper and lower respiratory tract infection. Respiratory syncytial virus -pneumonia was confirmed by PCR on nasopharyngeal aspiration. The child also had brief recent exposure to an adult TB source case; given the child's young age (<6 months) isoniazid preventive therapy (IPT) was prescribed. One gastric aspirate culture collected at enrolment grew M.tb after 42 days. At month 2, when the child was seen, the child was asymptomatic, growing well, and chest radiograph had resolved, so it was decided to continue with IPT only. Repeat gastric aspirates were negative; \*\*Not all HIV-infected children had documented perinatal exposure; §See Chapter 3 for comprehensive definition of antituberculosis regimen: regimens were categorised as "drug-resistant" regimen (DR-TB) if any of the medications typically given for the treatment of drug-resistant TB were given at any time during the first 2 months following enrolment, whether for treatment or prophylaxis. IQR: inter-quartile range; m: months; ART: antiretroviral therapy; VL: viral load; WFAZ: weight-for-age Z-score; HFAZ: height/length-for-age Z-score; TST: tuberculin skin test (Mantoux); CXR: chest X-ray; PTB: pulmonary TB; EPTB: extrapulmonary TB; DS-TB: drug-susceptible TB; DR-TB: drug-resistant TB.*

**Table 5.2. Baseline microbiological status of children enrolled with suspected pulmonary (intrathoracic) tuberculosis**

	<b>Microbiologically confirmed [n=117*, %]</b>
Xpert positive, culture positive	61 (52.1)
– Smear positive <sup>a</sup> :	21 (34.4)
○ Scanty positive	6 (28.6)
○ 1+ positive	9 (42.9)
○ 2+ positive	3 (14.3)
○ 3+ positive	3 (14.3)
– Lowest <sup>b</sup> culture TTP in days, median (IQR)	15 (10-21)
– Lowest <sup>c</sup> Xpert Ct value, median (IQR) [n=53]	26 (22-29.5)
– DST (LPA):	
○ RIF S INH S <sup>§</sup> / smear positive (%)	48 <sup>§</sup> (78.7)/15 (31.2)
○ RIF S INH R/ smear positive (%)	6 (9.8)/ 5 (83.3)
○ Rif R INH S/ smear positive (%)	4 (6.6)/0
○ RIF R INH R/ smear positive (%)	3 (4.9)/1 (33.3)
▪ AMIK R OFL R	1 (33.3)
Xpert negative, culture positive	40 (34.2)
– Smear positive	0
– Lowest <sup>b</sup> culture TTP	26 (21-31.5)
– DST (LPA):	
○ RIF S INH S	33 (82.5)
○ RIF S INH R	0
○ Rif R <sup>#</sup> INH S	1 (2.5)
○ RIF R INH R	5 (12.5)
▪ AMIK R OFL R	1 (20.0)
▪ AMIK S OFL R	1 (20.0)
○ No DST available	1 (2.5)
Xpert positive, culture negative	11 (9.4)
– Smear positive	0
– Lowest <sup>c</sup> Xpert Ct [n=9]	31 (29-33)
– DST:	
○ Xpert RIF S	8 (72.7)
○ Xpert RIF Indeterminate	3 (27.3)
Microbiology positive only on non-respiratory specimens <sup>d</sup>	
– FNAB <sup>e</sup>	3 (2.6)
– Stool <sup>f</sup>	2 (1.8)

*TTP: time to positivity for positive cultures; Ct: cycle threshold for Xpert; DST: drug susceptibility testing; RIF: rifampicin; S: susceptible; INH: isoniazid; R: resistant; AMIK: amikacin; OFL: ofloxacin; FNAB: fine-needle aspiration biopsy of a peripheral lymph node. \*Microbiological confirmation is defined as M.tb positive on either Xpert or culture on respiratory and non-respiratory specimens; <sup>§</sup>One child with rifampicin and isoniazid-susceptible TB on line probe assay had rifampicin resistance on Xpert which was confirmed on genotyping. The child was assessed as having possible hetero-resistance (see text for details); <sup>#</sup>this child was Xpert negative on respiratory specimens but had fine-needle aspiration biopsy of a peripheral lymph node which was Xpert positive rifampicin resistant.*

*One child, described in Table 5.1 above as a symptomatic control, had a gastric aspirate which was culture positive for M.tb after 42 days, but the child never received antituberculosis treatment and improved. Although there was “microbiological confirmation” of TB, there was doubt about lab contamination and the clinical importance of the result in the child who had completely recovered without antituberculosis treatment; for these reasons the child is not included in this table of microbiologically confirmed cases.*

*<sup>a</sup>The highest smear grade is reported to account for cases who may have had >1 smear-positive specimen; <sup>b</sup>This is the median of the lowest TTP value, to account for children who had >1 positive culture; <sup>c</sup>This is the median of the lowest mean cycle threshold, to account for children who had >1 positive Xpert result; <sup>d</sup>These children had respiratory specimens collected as per protocol, but all were microbiologically negative; all 5 cases had drug-susceptible TB; <sup>e</sup>In addition to these 3 children, another 7 had positive results on FNAB of peripheral lymph nodes (total number of children confirmed on FNAB=10; 8.5%), but they were also microbiologically confirmed on respiratory specimens; <sup>f</sup>One child had a positive Xpert result and one a positive culture on stool.*

**Table 5.3. Microbiological status by age group**

Age group	<6 m	7-12 m	13-24 m	25-36 m	37-60 m	>61m	Total
Total bacteriologically confirmed in age group	18 (100)	21 (100)	24 (100)	13 (100)	17 (100)	24 (100)	117 (100)
Xpert +, Culture +	16 (88.9)	12 (57.1)	9 (37.5)	5 (38.5)	7 (41.2)	12 (50.0)	61 (52.1)
Xpert -, Culture +	1 (5.6)	7 (33.3)	10 (41.7)	4 (30.8)	10 (58.8)	8 (33.3)	40 (35.0)
Xpert +, Culture -	1 (5.6)	2 (9.5)	4 (25.0)	3 (23.1)	0	1 (4.2)	11 (9.4)
Confirmed on non-respiratory specimens	0	0	1 (4.2)	1 (7.7)	0	3 (12.5)	5 (4.3)

*+: positive; - : negative; m: months*

### 5.3 Discussion

In this prospective cohort of children investigated for suspected PTB based on presenting symptoms and other clinical-epidemiological evidence, a diagnosis of PTB was made in almost one in two children (46%), suggesting that targeted investigation of high-risk children is effective in identifying a large proportion of children with active PTB. While clinically diagnosed cases constituted 58% of all children treated for TB, the proportion with a microbiologically confirmed diagnosis (>41%) is higher than that reported by many other published TB diagnostic studies, especially in young children, from South Africa and other high TB-burden settings<sup>123,124,212,213</sup>.

A number of factors may have contributed to this relatively high proportion of confirmed cases: as a hospital-based cohort, most children with PTB may have had more severe disease, resulting in higher bacillary burden. However, radiological severity of PTB was equally balanced between severe and non-severe disease, with 49% of children with radiological features typical of TB having radiologically severe TB (Table 5.1). Although the bacteriological yield was higher in the severe PTB group (76%), the yield was still substantial in children with non-severe PTB (40%). Secondly, the study applied rigorous eligibility criteria to identify children at highest risk of active TB. Thirdly, this was a diagnostic study, where particular focus was placed on trained study personnel collecting multiple respiratory specimens of different types (exceeding the local standard of care), following standard protocols with predefined minimum requirements for quality and volume of specimens. Increasing the number and type of specimens is known to result in higher detection of *M.tb* in children, who typically have paucibacillary disease<sup>107,108,126,214</sup>. Although

the strategy of collection of multiple specimens and specimen types is not likely to be feasible for routine care in many resource-limited settings, it adds strength to this study, as it has allowed me to study a representative sample of children with confirmed PTB, with a broad spectrum of PTB disease, in relation to clinically important covariates such as age and HIV infection. In addition, the study methods used strengthened the reference standard (microbiological confirmation including culture-confirmation) against which to evaluate the novel diagnostic strategies presented in the subsequent chapters of this dissertation.

The data from this study show that, although the majority of children presented with at least one of the well-defined symptoms of paediatric PTB, each individual symptom was present in similar proportions across all diagnostic groups (confirmed and clinical cases as well as symptomatic controls). This supports previous studies showing that the use of symptoms only has limited diagnostic utility to detect or exclude TB in children living in settings with high burden of respiratory infections including TB and chronic conditions such as malnutrition and HIV infection<sup>43,44,215,216</sup>. The study was however not designed to investigate the diagnostic utility of signs and symptoms for paediatric PTB.

TST positivity and history of TB exposure were significantly more common in children with microbiologically confirmed TB, than in those with a clinical diagnosis of PTB. Although this may indicate over-diagnosis among the clinically diagnosed cases, these findings may also reflect more intense exposure and higher “infecting dose” of *M.tb* experienced by children with microbiologically confirmed disease, potentially resulting in more rapid progression to disease<sup>217</sup>.

Chest radiography as a diagnostic tool was useful to detect PTB disease patterns associated with higher bacillary burden and to describe the spectrum and severity of disease, which, in subsequent chapters, are investigated in relation to microbiological detection. However, as a tool for detection of PTB disease, the CXR had important diagnostic limitations, despite our careful use with dual blinded standard reading. More than 13% of CXRs from symptomatic controls were reported as “typical of PTB”. Radiological manifestations typically associated with PTB in children, including mediastinal lymph nodes and pleural effusions, can occur with several other lung conditions <sup>200</sup>. In addition, the spectrum of radiologically typical features of paediatric PTB is not fully standardised and there is considerable variability in the definitions used by different diagnostic studies of paediatric PTB in the literature. Inter-observer variability in reporting has been described before, and adds to the limitations of using only chest radiography as a diagnostic tool for paediatric PTB <sup>93,218</sup>. Although my study had relatively strict criteria for defining radiologically typical PTB, there was evidently overlap with other respiratory conditions. The spectrum of conditions which mimic PTB radiologically in symptomatic children from high TB-burden settings is beyond the scope of this dissertation but requires further study. These data again also highlight the importance of the combined use of clinical symptoms, history including TB exposure history, chest radiography and bacteriology, to diagnose PTB in children.

Due to the inherent limitations of all the currently available tools to diagnose TB in children, a degree of over-diagnosis among clinical cases probably occurred, and is not unexpected from a setting where TB is highly endemic <sup>22</sup>. Over-diagnosis

based on clinical grounds in a certain proportion of children may in fact be seen as acceptable, and may be preferable to missing a diagnosis, particularly in children who are at high risk of progression to severe forms of TB, such as young and HIV-infected children. The exquisite vulnerability of very young children to progress to TB disease is well demonstrated in this cohort, where infants <6 months of age had a very high prevalence of severe pulmonary disease (>72% of typical CXRs were severe, 23% with cavities) and disseminated TB (18%). A high proportion of these infants was also HIV-exposed (30%), indicating the high risk of TB exposure early in life in settings with high burden of TB and HIV <sup>52,211,219</sup>. This was also observed in this cohort, with 44% of infants <6 months having reported close contact with an adult TB source case within the household. Microbiological measures of bacillary load also indicate that TB in these young infants had similar bacillary burden as that of children >5 years of age, of whom 10% had adult-type disease. Similarly, the microbiological disease status representative of the highest bacillary burden, Xpert positive and culture positive PTB, was most prevalent in infants <6 months (16/90: 17.8%) and in children >5 years old (12/60: 20%). Furthermore, 88.9% of infants <6 months old who had microbiologically confirmed PTB were in this “high bacillary load category”, compared to 50.0% of children with microbiologically confirmed PTB who were >5 years of age. In fact, cavitating lung disease and smear positivity displayed a bimodal distribution, with infants <6 months and children >5 years of age having the highest prevalence of both these attributes. A previous case series of 17 infants <6 months of age requiring mechanical ventilation for PTB in an Intensive Care Unit at TBH, Cape Town, described a similar spectrum of severe disease <sup>192</sup>. The high bacillary load in



these infants, together with the high prevalence of miliary TB seen in this group (19%), demonstrate the aggressive nature of TB disease in these youngest children. A review of 73 cases of microbiologically confirmed perinatal TB (infants <3 months of age) also documented a high proportion (>70%) of smear-positive TB, but the report included mostly children with confirmed TB, while this cohort enrolled a representative sample of children with clinically suspected PTB. Anatomical and immunological factors together place infants at high risk of complicated and disseminated TB. Interventions to promote early and effective TB prevention, together with sensitive and rapid diagnostic strategies, are urgently needed to reduce the extremely high burden of morbidity experienced by the youngest infants, many of whom were also HIV-exposed, which potentially affects mycobacterial immune responses early in life <sup>220</sup>. In addition, these data demonstrate that the youngest infants, contrary to widely held perceptions, are potentially a highly infectious group, and should be managed with strict observation of adequate infection control measures, particularly in hospitals and during collection of respiratory specimens.

HIV-infected children were identified as another specifically vulnerable group. Although this cohort was biased towards the inclusion of ill HIV-infected children with advanced immune compromise and poor viral control, regardless of ART, it is in fact this subgroup of HIV-infected children who is at highest risk of developing TB <sup>51,53,80</sup>. From a diagnostic perspective, the clinical presentation in HIV-infected children was similar to the rest of the cohort, including the prevalence of microbiologically confirmed TB. However, the radiological and microbiological spectrum of disease was different than that observed in HIV-uninfected children.

Similar to the youngest age group, HIV-infected children had significantly higher prevalence of miliary TB. In addition, HIV-infected children were significantly more likely to have involvement of more than one extrapulmonary site. However, the proportion of children with smear positivity was low among HIV-infected children (2%), as was the presence of cavities and complicated lymph node disease. This may be an indication that different immunological factors are at play in children who are immunologically compromised due to young age compared to those with impaired immunity due to HIV, or may also indicate earlier investigation of HIV-infected children for TB by health services, where TB screening is recommended before ART initiation in all HIV-infected patients. Finally, although the prevalence of confirmed TB among HIV-infected and HIV-uninfected children was similar, a significantly higher proportion of children in the clinically diagnosed group were HIV-infected. This reflects the challenges of diagnosis in the face of HIV-TB coinfection, the perceived high risk of TB in HIV-infected children, and possibly the lower threshold for prescribing antituberculosis treatment to HIV-infected children in this high-burden TB setting (as evidenced by the high proportion of HIV-infected children with a previous TB treatment episode). To best address the diagnostic needs of this vulnerable patient population, priority should be given towards the development of novel diagnostic tests which are not only more sensitive, but are also able to distinguish between TB, other common co-infections and chronic inflammatory conditions. This would reduce over-treatment, pill burden and potential toxicity from drug-drug interactions and side effects, in children already exposed to multiple chronic medications.

The detailed microbiology of TB described in this cohort (Table 5.2) provides interesting new insights into the microbiological spectrum of PTB disease in young children with respect to bacillary burden. The data clearly demonstrate that the group with respiratory specimens which were positive by both Xpert and culture had the highest bacillary load: almost a third of children in this group had smear-positive PTB disease, and the culture TTP and Xpert Ct values were significantly lower than those in the other groups. Although numbers are small, isoniazid-mono-resistant TB was prominent in this group. Isoniazid mono-resistance is associated with severe disease and poor outcomes in adults <sup>221-223</sup> and children <sup>224</sup>, if not appropriately treated, although the mechanisms underlying this are not well described and its optimal management is still uncertain <sup>225</sup>. Although the overall prevalence of isoniazid resistance in our setting has been stable in recent years at approximately 12% <sup>226</sup>, the severe clinical presentations of isoniazid-mono-resistant TB have only recently been appreciated <sup>223,225</sup>. The advanced presentation of children with isoniazid-mono-resistant TB may in part be due to delayed diagnosis (Xpert used routinely in adults with suspected PTB only screens for rifampicin-resistant TB): unless a child has been exposed to an adult with documented isoniazid-resistant TB, the child may receive IPT if active TB is not diagnosed. Isoniazid-mono-resistant TB would progress in spite of IPT, and may be detected only later if the child is subsequently microbiologically investigated. In this cohort, however, only one of the six children had an identified adult TB source case, who was however diagnosed with MDR-TB. None of the six isoniazid-mono-resistant cases had received IPT or previous antituberculosis treatment. Diagnostic tests which could enable early diagnosis and

rapid accurate DST for isoniazid as well as rifampicin, may have substantial benefit for both children and adults.

Data from this cohort further confirm that although Xpert can provide rapid detection of *M.tb* in a number of children, it is less sensitive than liquid culture, and a number of children will remain unconfirmed if culture is not available or not attempted. In this cohort, 35% of children were Xpert negative and detected only on culture, despite these children having been investigated with multiple Xpert tests on multiple specimens. This proportion is similar to what has been reported in the literature to date on the performance of Xpert in children <sup>25</sup> and highlights the challenges of applying diagnostic tests developed primarily for adult TB, to children who, for the most part, have paucibacillary disease. Children in the Xpert negative culture positive group had evidence of lower organism load than those in the Xpert positive culture positive group, shown by the significantly longer TTP for cultures. Relying on Xpert only would in addition have missed 25% of cases with DR-TB who were confirmed only by culture. Ensuring that culture is performed in child contacts of DR-TB adults is therefore critical in order to institute appropriate treatment.

A smaller proportion of children had *M.tb* detected only by Xpert. The longer Ct values observed in this group indicate a very low mycobacterial load in their respiratory specimens. It is possible that with very low bacillary concentrations, the laboratory decontamination methods currently followed (which are the same for adult sputum as for paediatric specimens) are too aggressive and may result in loss of viability of a critical number of bacilli <sup>134</sup>. Dead bacilli can be detected by molecular methods such as Xpert, but would not be recoverable on culture. Xpert was useful in

this small group, but the benefit of rifampicin resistance detection was limited, as 27% of test results had indeterminate rifampicin resistance. This was due to very low bacillary DNA concentrations resulting in probe delays.

An additional finding which has not previously been appreciated is that, when considering the group of children who were Xpert positive overall, a high proportion (21/82; 25.6% i.e. one in four) had smear-positive PTB. This underscores the need to enforce adequate infection control when managing children with Xpert-positive PTB, particularly in hospital and other in-patient facilities.

Finally, non-respiratory specimens, collected both routinely and for research, allowed for additional cases to be confirmed. The value of stool collection and testing for *M.tb* detection is investigated and presented in chapters 6 to 8. FNAB of peripheral lymph nodes remains a useful diagnostic procedure, as TB adenitis is the most common extrapulmonary manifestation of TB in children, and both traditional culture methods and Xpert typically have a rapid return of results if positive <sup>227</sup>.

In conclusion, I demonstrate that a high diagnostic yield was achieved using multiple specimen types tested by culture and Xpert, in a cohort of mostly young children with varied spectrum of PTB. An unexpectedly high proportion of young infants had severe PTB, including cavities, associated with high bacillary load. This calls for urgent priority to be given towards the development of tailored diagnostic tests that can rapidly confirm and quantitate *M.tb* disease in the youngest infants. This finding has major implications for the prompt treatment of young infants with adequate duration of appropriate antituberculosis regimens and dosages. Caution should be practiced before recommending shortened treatment regimens to young

infants. Strict infection control measures should be followed when managing and investigating young infants with suspected PTB. HIV-infected children had a high prevalence of disseminated TB and involvement of extrapulmonary sites, but had low prevalence of smear positivity.

## Chapter 6

# Stool as a specimen for the diagnosis of pulmonary (intrathoracic) tuberculosis in children using the Xpert MTB/RIF assay

### 6.1 Rationale

A major barrier preventing the microbiological investigation of children with suspected PTB is the current low capacity in routine care settings to collect appropriate respiratory specimens from young children who are unable to spontaneously expectorate sputum. This situation is particularly common in settings where TB is highly endemic and which bear the highest burden of paediatric TB <sup>228,229</sup>. In 2010, the WHO endorsed the Xpert MTB/RIF assay for the rapid diagnosis of TB and rifampicin resistance in adults, including in low-resource settings. At the time when I embarked on my study, in 2012, very limited data were available on the utility of Xpert for the diagnosis of paediatric TB, with only one published study reporting the performance of Xpert on IS collected in South African children <sup>124</sup>. The study found that Xpert on two IS specimens had a sensitivity of 75% compared to two cultures, with a high specificity of 98.8%. There were no published studies evaluating Xpert on GA or stool for diagnosing PTB in children. Indeed, the potential utility of stool as a diagnostic specimen for paediatric TB had not been thoroughly investigated before.

In young children, stool collection is potentially more feasible than collection of respiratory specimens, which requires trained health care workers and resources for collection and also careful consideration of infection control. Stool collection is also more child-friendly as it is not invasive. Nucleic acid amplification techniques are able to detect mycobacterial DNA in stool from swallowed sputum <sup>230</sup>. However, previous studies in children had documented low sensitivity (20-30%) <sup>107,116</sup> and specificity <sup>107</sup> of stool analysis by in-house mycobacterial PCR compared to culture of respiratory specimens.

## **6.2 Study Aims**

The aim of this prospective diagnostic study was to evaluate the diagnostic accuracy and clinical utility of Xpert on stool specimens for the diagnosis of PTB in a cohort of children with suspected PTB.

## **6.3 Methods**

I conducted an initial pilot study in 23 children with suspected PTB, which provided proof of concept for the use of Xpert both on GA and on stool specimens <sup>231</sup>: Xpert on stool and GA each detected 3 of 4 children with confirmed *M.tb* on GA culture. A prospective diagnostic cohort study was then implemented, in which children enrolled in the parent diagnostic cohort study described in detail in chapters 4-6, between April 2012 and August 2015, were included.



Specimen collection for smear microscopy, liquid culture and Xpert included one specimen each of GA in children <5 years of age or ESP in older children who could expectorate, IS and NPA collected daily over two days, and a stool specimen collected within 7 days of enrolment. Respiratory specimens were processed according to standard protocols at the NHLS at TBH. Stool specimens were processed by the study laboratory technician using two different methods, evaluated sequentially (not in parallel). For method 1, stool was homogenised with phosphate buffer solution in the laboratory, then decontaminated and concentrated prior to Xpert analysis. For method 2, the decontamination step was omitted: following initial concentration by centrifugation, the specimen pellet was re-suspended and a brief vortexing step was completed to remove large particulate matter. The resultant supernatant was mixed with Xpert standard reagent at the ratio recommended for respiratory specimens <sup>148,149</sup>.

The sensitivity and specificity of stool Xpert were compared to a) the overall yield by culture and Xpert on all respiratory specimens, and b) the yield of one Xpert test on GA or IS. In addition, clinical factors (covariates) associated with positive stool Xpert results were explored.

## **6.4 Results**

Three hundred and seventy nine children were included in this study: these were mostly young (median age 15.9 months) and 13.7% were HIV-infected. According to the NIH consensus case definitions <sup>174</sup>, 73 (19.3%) children had confirmed TB, 185 (48.8%) unconfirmed TB and 121 (31.9%) unlikely TB.

Microbiological confirmation was achieved in 73 (19.3%), of whom 14 (19.2%) had confirmed DR-TB. The sensitivity and specificity of stool Xpert vs. overall microbiological confirmation were 31.9% (95% CI 21.84-44.50%) and 99.7% (95% CI 98.2-100%), respectively. Stool Xpert sensitivity to detect rifampicin-resistant TB was 3/12 (25%). The two different stool processing methods had similar diagnostic accuracy, although stool processed by method 1 resulted in a higher proportion of invalid/error Xpert results (11.0%) compared to method 2 (3.7%;  $p=0.007$ ). Stool Xpert was positive in 23/51 (45.1%) children with radiologically severe PTB who were microbiologically confirmed on respiratory specimens. Cavities on CXR were associated with Xpert stool positivity after adjusting for age and other clinically relevant covariates (Odds ratio; OR 7.05; 95% CI 2.16-22.98;  $p=0.001$ ).

## 6.5 Conclusions

This was the first large study of Xpert testing of stool in children, which also evaluated clinical factors associated with positive test results on Xpert using stool specimens collected from children from a high-burden setting. Xpert testing of stool could rapidly confirm TB in children presenting with radiologic features suggestive of severe PTB. If processing of stool specimens could be simplified in future, Xpert on stool samples could improve access to rapid diagnostic confirmation and appropriate treatment for children from resource-limited settings where children frequently present with severe or advanced disease. In critically ill children, stool collection may be a safer alternative to invasive sputum collection procedures and should be considered as a potentially useful diagnostic strategy.

## 6.6 Citations

**Walters E**, Hesselning AC, Friedrich SO, Diacon AH, Gie RP. 2012. Rapid diagnosis of pediatric intrathoracic tuberculosis from stool samples using the Xpert MTB/RIF Assay: a pilot study. *Pediatr Infect Dis J* 31:1316.

**Walters E**, van der Zalm MM, Palmer M, Bosch C, Demers AM, Draper H, Goussard P, Schaaf HS, Friedrich SO, Whitelaw A, Warren R, Gie RP, Hesselning AC. 2017. Xpert MTB/RIF on Stool Is Useful for the Rapid Diagnosis of Tuberculosis in Young Children With Severe Pulmonary Disease. *Pediatr Infect Dis J* 36:837-843.

## 6.7 Candidate's Contribution

I was responsible for the design, implementation and data collection for this study. I designed the statistical analyses, interpreted the results, drafted and finalised the manuscript after feedback from the other co-authors.

## **6.8 Published Manuscripts**

## Rapid Diagnosis of Pediatric Intrathoracic Tuberculosis From Stool Samples Using the Xpert MTB/RIF Assay: A Pilot Study

### To the Editors:

Early rapid confirmation of tuberculosis (TB) in young children can prevent disease progression and death. The Xpert MTB/RIF assay (Xpert; Cepheid, Sunnyvale, CA), an automated real-time polymerase chain reaction assay, detects the presence of *Mycobacterium tuberculosis* in sputum within 2 hours. There are no published studies regarding the use of Xpert on stool or gastric aspirates (GA) for diagnosing pediatric intrathoracic TB. GA samples are commonly obtained from children with suspected TB in resource-limited settings; stool sampling is attractive as it is noninvasive.

From August to November 2011, we enrolled children less than 14 years of age with suspected intrathoracic TB from 2 hospitals in Cape Town in a cross-sectional pilot study. Suspected TB was  $\geq 1$  of the following: (1) symptoms suggestive of TB; (2) recent close contact with a TB source case; (3) reactive Mantoux skin test; (4) chest radiograph suggestive of TB, determined by 2 independent readers.<sup>1</sup> From each participant, 1 GA and 1 stool sample, if obtained, were analyzed by smear microscopy, culture and Xpert.

GA samples were neutralized with 4% sodium bicarbonate. Stool samples were homogenized by adding normal saline solution and by manual shaking. After decontamination and neutralization, 2 centrifugation steps of 20 minutes were performed. Supernatant was decanted; 30  $\mu$ L of the concentrated pellet was used for auramine O-microscopy.<sup>2</sup> The remaining pellet was resuspended in 1.5 mL phosphate buffer. Of this, 0.5 mL was inoculated into a supplemented Mycobacterial Growth Indicator Tube (Becton-Dickinson, Sparks, MD), and incubated at 37°C in a Bactec MGIT 960 machine. For Xpert, 0.7 mL of the resuspended pellet was mixed with 1.4 mL sample reagent and manually inverted before and during 15 minutes incubation. This mixture was transferred into 1 Xpert cartridge, loaded into the GeneXpert machine and processed automatically.

Of 28 eligible children, we included 23 (15 female, 2 HIV-infected, median age 17.2 months). In 5, we failed to obtain consent and/or samples before discharge. Stool and GA samples were obtained from 14 children; 6 had only GA and 3 had only stool collected. Twelve children received a diagnosis of TB, of whom 6 were culture-confirmed and 2 had extrathoracic TB. Overall, Xpert on stool and GA detected 3 of 4 (75%) children with intrathoracic TB and positive GA cultures, and 3 of 6 (50%) children with *M. tuberculosis* cultured from any site. Time to culture positivity was 5–18 (GA) and 22–32 (stool) days. No contaminated cultures, indeterminate Xpert results or drug resistance were reported.

This study suggests that Xpert on stool might be useful for the diagnosis of pediatric intrathoracic TB, as Xpert on stool and GA was equally sensitive and gave more rapid results than culture. We used longer initial centrifugation times to increase sample concentration and to improve the detection of *M. tuberculosis* in stool. Stool sampling is “child-friendly” and has minimal infectious risk, overcoming some barriers to bacteriologic investigation of TB in children. Rapid confirmation using noninvasive sampling may be particularly useful in young children with extensive TB or suspected drug resistance, and its potential clinical impact should be systematically investigated.

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## Foul Smelling Urine in a 7-year-old Boy Caused by *Aerococcus urinae*

### To the Editors:

*Aerococcus urinae* is an infrequently reported Gram-positive coccus mostly associated with urinary tract infection in elderly females. Sporadically, the organism has been described as the cause of severe infections.<sup>1</sup> We report a young boy who had malodorous urine as the only sign of colonization with *A. urinae*.

This 7-year-old boy was seen at the outpatient clinic because he exuded a bad odor. He was in good health and his medical history was unremarkable. Urinating was not painful and he had no fever. His mother described that after the boy urinated the whole house smelled bad. The general practitioner had found no leucocyturia or nitrite in the urine. Because it was believed that the smell occurred from smegma from under the preputium, a circumcision had been performed but this had no effect on the odor.

At physical examination, he was a healthy boy with normal weight and height. The examination, including the genitals, was normal. A metabolic screen was reported normal. Repeated urine cultures consistently revealed significant growth ( $10^5$  colony forming units/mL) of alpha hemolytic colonies identified as *A. urinae*. A course of 7 days treatment with co-trimoxazole was given and the smell disappeared. A follow-up urine culture was negative. Echography of the urinary tract revealed no abnormalities.

This boy had repeatedly *A. urinae* in his urine with a foul smell as the only sign. A single course of antibiotics totally resolved the problem of the malodor.

*A. urinae* is reported in about 0.2–0.3% of urine specimens sent in for culture. More than 90% of the patients are females >65 years of age. Most patients have a local or systemic underlying condition, predisposing to urinary tract infection.<sup>1</sup> There is no relation between odor and urinary tract infections in the elderly.<sup>2</sup> In young patients, *A. urinae* is reported rarely; we found only 1 article reporting pyelonephritis in a 12-year-old boy who had a history of pyeloplasty for vesicoureteral reflux.<sup>3</sup> Our patient had no signs of an underlying disease.

Although co-trimoxazole resistance has been described as a key feature in the identification of *A. urinae*, it was found recently that this is an *in vitro* effect. Most strains are susceptible to co-trimoxazole.<sup>4</sup>

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# Xpert MTB/RIF on Stool Is Useful for the Rapid Diagnosis of Tuberculosis in Young Children With Severe Pulmonary Disease

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**Background:** Tuberculosis (TB) continues to result in high morbidity and mortality in children from resource-limited settings. Diagnostic challenges, including resource-intensive sputum collection methods and insensitive diagnostic tests, contribute to diagnostic delay and poor outcomes in children. We evaluated the diagnostic utility of stool Xpert MTB/RIF (Xpert) compared with bacteriologic confirmation (combination of Xpert and culture of respiratory samples).

**Methods:** In a hospital-based study in Cape Town, South Africa, we enrolled children younger than 13 years of age with suspected pulmonary TB from April 2012 to August 2015. Standard clinical investigations included tuberculin skin test, chest radiograph and HIV testing. Respiratory samples for smear microscopy, Xpert and liquid culture included gastric aspirates, induced sputum, nasopharyngeal aspirates and expectorated sputum. One stool sample per child was collected and tested using Xpert.

**Results:** Of 379 children enrolled (median age, 15.9 months, 13.7% HIV infected), 73 (19.3%) had bacteriologically confirmed TB. The sensitivity and specificity of stool Xpert versus overall bacteriologic confirmation were

31.9% [95% confidence interval (CI): 21.84%–44.50%] and 99.7% (95% CI: 98.2%–100%), respectively. A total of 23/51 (45.1%) children with bacteriologically confirmed TB with severe disease were stool Xpert positive. Cavities on chest radiograph were associated with Xpert stool positivity regardless of age and other relevant factors [odds ratios (OR) 7.05; 95% CI: 2.16–22.98;  $P = 0.001$ ].

**Conclusions:** Stool Xpert can rapidly confirm TB in children who present with radiologic findings suggestive of severe TB. In resource-limited settings where children frequently present with advanced disease, Xpert on stool samples could improve access to rapid diagnostic confirmation and appropriate treatment.

**Key Words:** tuberculosis, diagnosis, children, stool

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Despite being preventable and treatable, tuberculosis (TB) contributed to at least 169,000 deaths among children in 2015.<sup>1</sup> Tuberculosis-related mortality and morbidity in children are likely underestimated, because of challenges in diagnosing TB in children and incomplete reporting. Tuberculosis frequently presents with nonspecific symptoms, especially in young and HIV-infected children, and is a frequent comorbid condition in children presenting with pneumonia and other respiratory childhood illnesses.<sup>2,3</sup> Tuberculosis has been reported in up to 23% of children with severe pneumonia in high TB-burden settings,<sup>2</sup> with autopsy studies from African urban hospitals identifying TB in 4%–20% of pediatric deaths, the majority undiagnosed.<sup>3–5</sup> Although early diagnosis and treatment of TB in children is associated with excellent treatment outcomes,<sup>6,7</sup> the majority of children with TB live in low-resource settings with poor access to timely TB diagnosis and intervention.<sup>8,9</sup>

Tuberculosis in children is mostly intrathoracic (pulmonary) in >75% of cases,<sup>10,11</sup> and is mainly paucibacillary (smear-negative). Current bacteriologic tests to confirm TB in children have limited sensitivity and resource-intensive methods are needed to collect adequate samples. Current available tests confirm a minority of children with TB: 5%–10% of children with TB are smear microscopy positive and 10%–40% culture positive depending on disease severity and spectrum,<sup>12,13</sup> resulting in a perceived unfavorable cost-benefit ratio for bacteriologic testing in most resource-limited settings. The Xpert MTB/RIF<sup>®</sup> assay (Xpert; Cepheid, Sunnydale, CA), endorsed by the World Health Organization for the diagnosis of TB and rifampicin resistance in adults and children including in resource-limited settings in 2010,<sup>14</sup> yields rapid results, but has <70% sensitivity compared with culture in children.<sup>15</sup> Xpert also requires appropriate samples in young children, such as gastric aspirates (GA), induced sputum (IS), nasopharyngeal aspirates, fine needle aspiration of peripheral lymph nodes or bronchoalveolar lavage,<sup>13,16</sup> all resource-intensive, invasive procedures.

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Access to rapid diagnosis for childhood TB requires more than the availability of diagnostic tests. Bacteriologic diagnosis will remain largely confined to referral hospitals or well-resourced settings unless tests can be applied to samples that are easy to collect at the level of the healthcare system where children initially present, typically the primary healthcare level in resource-limited settings.<sup>17</sup> The evaluation of any new diagnostic strategy for childhood TB should therefore consider not only diagnostic accuracy but also the potential application of such strategies at relevant levels of healthcare.<sup>18–20</sup>

Stool collection is potentially a more feasible and acceptable alternative to collecting respiratory samples, and can occur at home. *Mycobacterium tuberculosis* (*M.tb*) in swallowed sputum is recoverable from stool samples by culture or molecular methods.<sup>21–23</sup> Stool does not require sophisticated collection equipment and poses low TB infection risk to healthcare workers. In particular, stool is easy to obtain from young children, a high-risk group for severe TB and where diagnosing TB is the most challenging. In critically ill children, stool collection may be a safer alternative to invasive sputum collection procedures.

In a prospective hospital-based study of children presenting with suspected intrathoracic TB in Cape Town, South Africa, we evaluated the diagnostic accuracy of Xpert on stool samples compared with bacteriologic confirmation from respiratory samples, and also to a clinical reference standard. We determined which clinical and bacteriologic factors were associated with positive stool Xpert.

## MATERIALS AND METHODS

### Eligibility

Children <13 years of age who presented consecutively to 2 hospitals (Tygerberg Hospital and Karl Bremer Hospital, Cape Town, South Africa) with suspected intrathoracic TB, were screened for enrolment from April 2012 to August 2015. The enrolling facilities provide primary to tertiary level medical services to approximately 30% of the total Cape Town population of approximately 4 million. Children ≤13 years of age were eligible, if they presented with ≥1 of (1) cough ≥2 weeks, (2) unexplained fever ≥1 week or (3) poor growth or weight loss over the preceding 3 months. With consideration of the fact that TB may present acutely, especially in young children, we also included children with any duration of cough, if ≥1 of the following were present: (1) exposure to an identified TB source case in the past 12 months, (2) positive tuberculin skin test if previously negative or unknown or (3) a chest radiograph (CXR) suggestive of TB as assessed by the study clinician. Infants <3 months of age were also eligible if they had pneumonia unresponsive to appropriate antimicrobials, or unexplained and unresponsive sepsis syndrome. We excluded children who had received >1 dose of antituberculosis therapy (excluding isoniazid preventive therapy), had an established alternative diagnosis at screening, or who resided in areas too remote to ensure retention.

### Clinical Investigations

Clinical investigations followed established local and international practice,<sup>24</sup> including a detailed history of symptoms and TB exposure, a clinical examination, tuberculin skin test (Mantoux, 2 Tuberculin Units PPD RT-23, Statens Serum Institute, Copenhagen), CXR (antero-posterior and lateral films) and a minimum of 2 respiratory samples for TB bacteriology (see section “Laboratory Methods”). Chest radiograph were read by 2 independent experts, using a standard reading tool. Consensus was determined on the certainty of TB diagnosis, the radiologic pattern and extent of disease. Discrepant readings were resolved by a third reader or by a

forum if consensus was not reached. Disease severity was determined using a pragmatic modification of a published classification.<sup>25</sup> Radiologically severe TB was defined as any of (1) evidence of complications resulting from typical radiologic manifestations of TB (eg, cavities, expansile pneumonia, nodal airway obstruction), (2) bilateral parenchymal involvement, (3) overall parenchymal involvement more extensive than the total area of the right upper lobe or (4) disseminated (miliary) TB. For CXR not typical of TB, criteria 2 and 3 were used to define severe disease.

Any additional imaging results (eg, abdominal ultrasound, chest computed tomography) were incorporated into the final classification to describe the full spectrum of disease. Disseminated disease was defined as miliary TB or neurotuberculosis.

The final classification of the certainty of TB disease was determined retrospectively at the 2-month follow-up, when final culture results from enrolment were available and response to treatment was evaluated. Participants were classified using the revised clinical case definitions for diagnostic research of intrathoracic TB in children,<sup>20</sup> into the categories “confirmed TB,” “unconfirmed TB” and “unlikely TB.” The decision to treat for TB was made by the attending clinicians, based on clinical/epidemiologic assessment and the results of all investigations (not based on research case definitions). Xpert results from study samples, including stool, were available to clinicians and may have influenced treatment decisions. However, this study did not evaluate the impact of Xpert results on decision to treat. All children were followed for 6 months by the study team regardless of final TB diagnosis, to assess clinical course and treatment response. Children with drug-resistant TB were followed to treatment completion.

### Sample Collection

The complete sample schedule according to the study protocol consisted of 1 sample each of GA (in children <5 years of age)/sputum (SPT) (older children who could expectorate), IS and nasopharyngeal aspirates (Supplemental Digital Content 1, <http://links.lww.com/INF/C683>) collected daily for 2 consecutive days, and a stool sample (maximum total 7 samples). Stool was collected within 7 days of enrolment. Some children also had fine needle aspiration of peripheral lymph nodes and bronchoalveolar lavage, collected by the hospital staff, if clinically indicated. If any of the samples required by the study schedule were collected by hospital personnel, these were not collected again by the research team; their bacteriologic results were documented.

### Laboratory Methods

Fluorescent smear microscopy, liquid culture and Xpert were completed on all respiratory samples collected by the research team. Because of cost constraints, samples collected by hospital personnel were generally only tested by smear and culture.

After collection, GA samples were titrated to neutral pH using 4% sodium bicarbonate solution.<sup>24</sup> All respiratory specimens were kept refrigerated and transported to the laboratory in a cool box within 4 hours of collection. Stool samples collected at home from children discharged from hospital and those that could not be processed immediately were stored at 2–8°C for maximum 72 hours before processing.

Respiratory specimens were handled by the National Health Laboratory Service at Tygerberg Hospital. In brief, after decontamination with N-acetyl-L-cysteine-1.25% sodium hydroxide, neutralization and concentration, the pellet was resuspended in 1.5 mL phosphate buffered saline (PBS). The sample was utilized for Auramine-O smear microscopy,<sup>26</sup> liquid culture using the mycobacteria growth indicator tube system (Becton Dickinson, Sparks, MD)<sup>27</sup> and Xpert. Positive cultures were confirmed for the presence

of *M.tb* by Ziehl-Neelsen staining and microscopy, absence of any growth on blood agar plates and assessed for resistance against rifampicin and isoniazid using the GenoType MTBDR<sub>plus</sub> line probe assay (Hain Life Science, Nehren, Germany). Rifampicin-resistant isolates underwent second-line drug-susceptibility testing for amikacin and ofloxacin using the agar proportion method.

Stool specimens were processed by the study laboratory technician. In the absence of Xpert manufacturer specific instructions for stool, 2 methods were used (adapted from Refs. 28, 29). For method 1, up to 5 g of stool was homogenized with 20 mL PBS by vortexing; 5 mL of the stool/PBS mixture was then processed as above with N-acetyl-L-cysteine-1.25% sodium hydroxide. After concentration, 0.5 mL of the resuspended pellet was used for mycobacteria growth indicator tube culture and 1.0 mL was mixed with 2.0 mL of Xpert sample reagent, and loaded into the GeneXpert instrument (Software Version 4.4a) following manufacturer instructions.

In June 2014, stool culture was discontinued because of high contamination and low sensitivity and stool method 2 was implemented: 1–4 g of specimen was homogenized with 10 mL PBS. After centrifugation at 3000×g, 4°C for 20 minutes, the supernatant was discarded and the pellet resuspended in 10 mL of PBS by vortexing for 20 seconds. Having settled large particulate matter by brief centrifugation (30 seconds; 2000×g), 1 mL supernatant was mixed with 2 mL Xpert sample reagent and tested by Xpert.

This study was approved by the Stellenbosch University Health Research Ethics Committee (reference N11/09/282), by the participating hospitals and provincial department of health. Written informed consent for study participation was obtained from parents or legal caregivers, and written assent was obtained from older children.

### Statistical Analysis

The primary objective of this study was to evaluate the sensitivity, specificity and predictive values of stool Xpert for the diagnosis of intrathoracic TB in children, compared with (1) overall bacteriologic confirmation and (2) a clinical reference standard. The bacteriologic reference standard was defined as confirmation of *M.tb* by culture or Xpert of any samples collected by the research team or hospital personnel, including stool culture but excluding stool Xpert (index test and therefore not included in the reference standard). Not all children had all samples collected: children were included in analysis if they had a minimum of one stool and one respiratory sample collected (intention to diagnose analysis). All analyses of diagnostic accuracy were conducted per patient (not per sample). The reference was considered positive if any of the samples collected from any individual child were *M.tb* positive on Xpert or culture. The reference was considered negative if none of the samples collected from any individual child were positive for *M.tb* on Xpert or culture. Nonevaluable results (invalid/error Xpert results) were reported, and were considered negative for the diagnostic accuracy analysis if the child had at least one other valid result.

We also compared the diagnostic accuracy of stool Xpert to the first GA and IS Xpert, with the first GA or IS culture as a reference, respectively, to better assess the value of stool Xpert compared with a single respiratory sample.

The clinical reference standard was defined as the clinician decision to treat for TB, where a “TB case” was defined as a child in whom antituberculosis treatment was initiated, and a “symptomatic control” was a child investigated for TB based on the same entry criteria, but who did not initiate treatment.

In addition, we report on the proportion of children with positive stool Xpert by international consensus case definitions of

“confirmed,” “unconfirmed” and “unlikely” TB.<sup>20</sup> According to this classification, “confirmed TB” is defined as bacteriologic confirmation by culture or Xpert of *M.tb* on any valid respiratory sample, including stool. We therefore included all children with any positive Xpert or culture result on any sample in this category (including stool Xpert).

Clinical and demographic characteristics were summarized by clinical case definitions using means and standard deviations if normally distributed and with medians and interquartile ranges if non-normally distributed.<sup>20</sup> STARD guidelines were followed for reporting and analyses.<sup>30</sup>

Univariable analysis was used to identify factors associated with Xpert-positive stool. Any factors associated at a *P* value <0.10 were used to build a multivariable logistic regression model. For multivariable regression and comparative analyses, odds ratios (OR) and 95% confidence intervals (CIs) were reported, with *P* values; a *P* value <0.05 was considered statistically significant. Analyses were generated using Stata 14.0 special edition software (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX, StataCorp LP).

### RESULTS

Stool was collected and tested by Xpert in 379 children (Fig. 1). According to research case definitions,<sup>20</sup> 73 children had confirmed TB, 185 unconfirmed TB and 121 unlikely TB. The median age was 15.9 [interquartile range (IQR) 9.2–29.3] months, the HIV prevalence was 13.7% and 27 (7.1%) children had a documented previous TB episode (Table 1).

### Spectrum of Disease Among TB Cases

Antituberculosis treatment was initiated in 170/379 (44.9%) children within 2 weeks of enrolment. In 164/170 (96.5%) children with CXR available for dual reading, 84/164 (51.2%) had radiologic patterns typical of TB, whereas 63/164 (38.4%) had nontypical patterns. Nine (5.5%) CXR were normal and in 8 (4.9%) the quality of the CXR was inadequate for evaluation. The most common radiologic pattern reported as typical of TB was intrathoracic lymph node disease in 64/164 (39.0%). Other typical patterns observed alone or in combination, were 17 with cavitating disease, 9 miliary, 8 expansile pneumonia, 4 Ghon complex and 2 pleural effusions. The most common nontypical changes were suggestive of lower respiratory tract infection (bronchopneumonia, lobar pneumonia and interstitial pattern) in 47 (28.7%) children. Overall, 84/164 (51.2%) TB cases had severe disease by CXR findings.

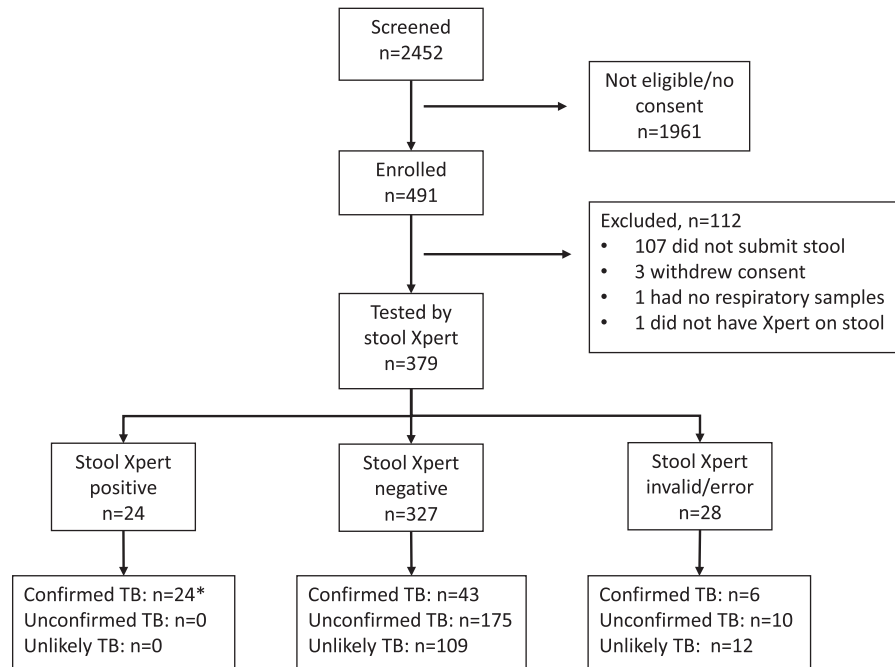
Thirty-five (20.6%) children also had extrathoracic TB: 15 disseminated (5/15 also had neurotuberculosis, 2/15 also abdominal TB and 1/15 also cervical TB adenitis), 11 abdominal TB and 9 cervical TB adenitis.

A total of 24/170 (14.1%) children were treated for drug-resistant TB: 16 were bacteriologically confirmed (6 multidrug resistant, 4 isoniazid monoresistant, 4 rifampicin monoresistant and 2 extensively drug resistant); 8 were empirically treated for multidrug-resistant TB based on symptoms, suggestive CXR and close contact with a confirmed multidrug-resistant TB source case.

### Bacteriology Results and Diagnostic Utility of Stool

In total, 1849 respiratory samples were collected (median samples/child = 4; range: 1–10). Of 73/170 (42.9%) children with bacteriologically confirmed TB, 71 were detected on culture or Xpert of nonstool samples, one only on stool culture, and one only on stool Xpert. A total of 11/73 (15.1%) children had smear-positive TB; these were all also positive on Xpert and culture of respiratory samples. Nine of 11 (81.8%) were stool Xpert positive, 1





\*One child was positive on stool Xpert, but all other samples were negative by Xpert and culture

**FIGURE 1.** Study profile, including stool Xpert results compared with clinical consensus case definitions for pulmonary TB in children.<sup>20</sup>

**TABLE 1.** Cohort Characteristics Overall and by International Consensus Diagnostic Category,<sup>20</sup> in Children Presenting With Suspected TB (N = 379)

Characteristic	All Children	Confirmed TB	Unconfirmed TB	Unlikely TB
	N = 379 (100%)	N = 73	N = 185	N = 121
Age, months: median (IQR)	15.7 (9.2–29.4)	19.1 (10.9–44.0)	16.0 (9.6–28.3)	13.3 (6.2–25.7)
Male (%)	195 (51.5)	27 (37.0)	105 (56.8)	63 (52.1)
Ethnicity				
Mixed race (%)	215 (56.7)	42 (57.5)	111 (60.0)	62 (51.2)
Black African (%)	163 (43.0)	31 (42.5)	73 (39.5)	59 (48.8)
Caucasian (%)	1 (0.3)	0	1 (0.5)	0
Perinatal HIV exposure (%)	105 (27.7)	18 (24.7)	54 (29.2)	33 (27.3)
HIV infected (%)	51 (13.5)	8 (11.0)	30 (16.2)	13 (10.7)
On ART at presentation (%)	22 (43.1)	4 (50.0)	14 (46.7)	4 (30.8)
Previous TB treatment (%)	27 (7.1)	4 (5.5)	18 (9.7)	5 (4.1)
Intrathoracic (%)	19 (70.4)	2	13	4
Extrathoracic/disseminated (%)	5 (18.5)	2	2	1
Unknown site of TB (%)	3 (11.1)	0	3	0
Completed previous TB treatment* (%)	24 (88.9) (n = 27)	4 (100) (n = 4)	15 (83.3) (n = 18)	5 (100) (n = 5)
WAZ, median (IQR)	-1.8 (-2.9 to -0.7)	-1.7 (-2.9 to -0.7)	-2.2 (-3.2 to -0.9)	-1.4 (-2.1 to -0.7)
HAZ, median (IQR)	-1.7 (-2.9 to -0.7)	-1.7 (-2.8 to -0.6)	-2 (-3.1 to -0.9)	-1.4 (-2.5 to -0.5)
WAZ < -2 (%)	168 (44.3)	31 (42.5)	103 (55.7)	34 (28.1)
Evidence of BCG immunization† (%)	360 (95.0)	65 (89.0)	178 (96.2)	117 (96.7)
≥1 well-defined TB symptom <sup>20</sup> (%)	310 (81.8)	66 (90.4)	169 (91.4)	75 (62.0)
TST positive‡ (%)	82 (28.0) (n = 293)	39 (72.2) (n = 54)	38 (26.2) (n = 145)	5 (5.3) (n = 94)
Exposure to identified TB source case (%)	214 (56.5)	51 (69.9)	141 (76.1)	22 (18.2)
CXR suggestive of TB§ (%)	105 (28.3) (n = 371)	51 (71.8) (n = 71)	47 (25.7) (n = 183)	7 (6.0) (n = 117)
Treated for TB (%)	170 (44.9)	73 (100)	69 (37.3)	28 (23.1)

\*Three children had missing/incomplete records of the reported previous TB episode, so site of TB and treatment outcome could not be determined.

†Either documented BCG vaccination in the Road To Health Card or BCG scar present.

‡≥10 mm if HIV negative and BCG vaccinated, ≥5 mm if HIV positive or not BCG vaccinated.

§As determined retrospectively by 2 independent expert readers.

ART indicates antiretroviral therapy; BCG, bacille Calmette–Guerin; CXR, chest radiograph; HAZ, height-for-age Z-score according to UK growth charts 1990; TST, tuberculin skin test; WAZ, weight-for-age Z-score according to UK growth charts 1990.

(9.1%) was stool Xpert negative and another (9.1%) had an invalid stool Xpert result, which was not repeated.

Among the 170 TB cases (ie, children treated for TB), children >5 years of age had a higher proportion of confirmed TB (13/18; 72.2%) compared with younger children (60/152; 39.5%,  $P = 0.008$ ). A total of 8/30 (26.7%) HIV-infected children were bacteriologically confirmed, compared with 65/140 (46.4%  $P = 0.047$ ) HIV-uninfected children, but a higher proportion of HIV-infected children overall initiated antituberculosis treatment (30/51; 58.8% vs. 140/328; 42.7%, respectively,  $P = 0.031$ ).

Stool Xpert detected 24 TB cases, of which 23 were also bacteriologically confirmed on non-stool samples. The sensitivity and specificity of stool Xpert versus overall bacteriologic confirmation were 31.9% (95% CI: 21.4%–44.0%) and 99.7% (95% CI: 98.2%–100%), respectively (Table 2). Stool Xpert detected rifampicin resistance in 3/12 (25%) confirmed rifampicin-resistant cases. Invalid/error Xpert results occurred in 17/1611 (1.1%) respiratory samples versus 28/379 (7.4%) stool samples: in 3/28 cases with invalid stool results, a second stool sample yielded negative results. Stool processed by method 1 versus method 2 yielded 21/191 (11.0%) versus 7/188 (3.7%) invalid/error Xpert results on the first stool tested ( $P = 0.007$ ).

Compared with the first GA culture, the sensitivity and specificity of stool Xpert were 45.5% (28.1–63.6) and 97.4% (98.3–100), whereas GA Xpert sensitivity and specificity were

54.5% (36.4–71.9) and 98.7% (96.2–99.7), respectively (Table 3). GA Xpert had an incremental detection of 3/33 (9.1%) over GA culture, whereas stool Xpert detected an additional 6/33 (18.2%).

Compared with the first IS culture, the sensitivity and specificity of stool Xpert were 50.0% (29.1–70.9) and 97.6% (94.8–99.1), whereas IS Xpert sensitivity and specificity were 66.7% (44.7–84.4) and 97.6% (94.8–99.1), respectively (Table 3). IS Xpert had an incremental detection of 6/24 (25.0%) over IS culture, whereas stool Xpert detected an additional 8/24 (33.3%).

**Spectrum of Disease in Stool-Xpert Positive Cases**

Radiologically severe disease was observed in all 24 stool-Xpert positive cases: 12 (52.2%) had complicated lymph node disease, 9 (39.1%) had cavitating disease (2 also with miliary disease and 2 with expansile pneumonia) and 1 more each had miliary TB and expansile pneumonia, respectively. One had bilateral alveolar opacification not typical of TB. Of the 51 bacteriologically confirmed cases with severe intrathoracic disease, 23 (45.1%) were stool Xpert positive.

Only 1 of 13 cases with abdominal TB accompanying intrathoracic TB had positive stool Xpert.

**Factors Associated With Xpert-positive Stool**

Univariable analysis of clinical features and their association with Xpert-positive stool is shown in Table 4. Cavities on CXR

**TABLE 2.** Diagnostic Accuracy of Stool Xpert Compared With Xpert and Culture of Respiratory and Non-Respiratory Samples (per Patient Analysis), and Compared With a Clinical Decision to Treat

	Sensitivity (%; 95% CI)	Specificity (%; 95% CI)	PPV (%; 95% CI)	NPV (%; 95% CI)
a. Diagnostic accuracy of stool Xpert compared with Xpert and culture of respiratory and non-stool non-respiratory samples				
Stool Xpert	23/72 (31.9; 21.4–44.0)	306/307 (99.7; 98.2–100)	23/24 (95.8; 78.9–99.9)	306/355 (86.2; 82.2–89.6)
b. Diagnostic accuracy of stool Xpert compared with clinical decision to treat				
Stool Xpert	24/170 (14.1; 9.3–20.3)	209/209 (100; 98.3–100)	24/24 (100; 85.8–100)	209/355 (58.9; 53.6–64.0)

NPV indicates negative predictive value; PPV, positive predictive value.

**TABLE 3.** Diagnostic Accuracy of Stool Xpert Versus the First Gastric Aspirate or Induced Sputum Sample

	Sensitivity (%; 95% CI)	Specificity (%; 95% CI)	PPV (%; 95% CI)	NPV (%; 95% CI)
a. Diagnostic accuracy of the first GA Xpert compared with the first GA culture				
GA Xpert	18/33 (54.5; 36.4–71.9)	226/229 (98.7; 96.2–99.7)	18/21 (85.7; 63.7–97.0)	226/241 (93.4; 89.9–96.5)
b. Diagnostic accuracy of stool Xpert compared with the first GA culture				
Stool Xpert	15/33 (45.5; 28.1–63.6)	223/229 (97.4; 98.3–100)	15/21 (71.4; 47.8–88.7)	223/241 (92.5; 88.5–95.5)
c. Diagnostic accuracy of the first IS Xpert compared with the first IS culture				
IS Xpert	16/24 (66.7; 44.7–84.4)	240/246 (97.6; 94.8–99.1)	16/22 (72.7; 49.8–89.3)	240/248 (96.8; 93.7–98.6)
d. Diagnostic accuracy of stool Xpert compared with the first IS culture				
Stool Xpert	12/24 (50.0; 29.1–70.9)	240/246 (97.6; 94.8–99.1)	12/18 (66.7; 41.0–86.7)	240/252 (95.2; 91.8–97.5)

NPV indicates negative predictive value; PPV, positive predictive value.

**TABLE 4.** Univariate and Multivariate Analysis Using Logistic Regression to Determine Characteristics Associated With Positive Stool Xpert (N = 170)

Factor	Univariable OR (95% CI)	P	Multivariable OR (95% CI)	P
Age > 5 years	2.69 (0.86–8.40)	0.088	2.04 (0.56–7.47)	0.280
Male sex	0.75 (0.31–1.81)	0.528		
Chest radiograph: cavities	10.35 (3.48–30.82)	<0.001	7.05 (2.16–22.98)	0.001
Chest radiograph: miliary	3.33 (0.77–14.35)	0.106		
Chest radiograph: expansile pneumonia	7.10 (1.64–30.66)	0.009	3.97 (0.73–21.71)	0.112
Black ethnicity	0.61 (0.24–1.50)	0.280		
HIV exposed (n = 166)	0.77 (0.28–2.08)	0.604		
HIV infected	0.63 (0.18–2.26)	0.479		
WAZ < -2	1.56 (0.65–3.71)	0.317		
Any antibiotic treatment before TB sample collection	0.72 (0.30–1.71)	0.453		
Stool processing method 2 vs. method 1*	1.47 (0.62–3.51)	0.381		

\*See section "Laboratory Methods."

WAZ indicates weight-for-age Z-score according to UK growth charts 1990.

and expansile pneumonia were associated with having positive stool Xpert. HIV status, malnutrition, antibiotics before sample collection and stool processing method were not significantly associated with stool Xpert status. In multivariable logistic regression, adjusting for age and expansile pneumonia, cavities remained significantly associated with positive stool Xpert (OR 7.05; 95% CI: 2.16–22.98;  $P = 0.001$ ).

## DISCUSSION

To our knowledge, this is the largest study to investigate the value of Xpert on stool samples for the diagnosis of TB in HIV-uninfected and HIV-infected children. We explored not only the diagnostic accuracy but also the clinical applicability of stool Xpert. Our study population is representative of children with suspected TB from many high-burden settings, who are frequently referred to hospital for TB investigation. A large proportion of children presenting in this manner already have advanced TB disease, often with comorbidity and coinfections,<sup>2,3,31</sup> further compounding diagnostic challenges. In high-burden TB settings, resource-intensive methods for the collection of respiratory samples from young children and the relatively low yield of diagnostic tests in this population often pose a barrier to attempting diagnostic confirmation both at the community-based clinic and at referral level.

Xpert testing of stool is likely to have the greatest impact in settings where TB in children is typically diagnosed on clinical grounds, and where diagnosis is therefore frequently delayed or missed.<sup>32</sup> This is more common in very young children and in those with HIV coinfection, who may present acutely, with non-specific signs and symptoms,<sup>32,33</sup> and with severe disease, and who are at high risk of dying with undiagnosed TB.<sup>3,5</sup> The majority of children in our study belonged to these high-risk groups. Applied to this patient population which is at exquisitely high risk of rapid disease progression and poor clinical outcomes, stool Xpert may therefore potentially be a life-saving strategy, particularly where capacity for respiratory sample collection, culture and drug-susceptibility testing is limited. Furthermore, for children exposed to drug-resistant TB, rapid confirmation of TB and drug resistance is likely to improve access to timely and appropriate treatment. Our study indicates that targeting Xpert testing of stool in severely ill children referred to hospital with suspected severe intrathoracic TB has a high yield. If stool processing could be simplified and the diagnostic performance optimized, stool Xpert could be promoted for use in clinic settings where children have the first contact with the healthcare system.

Published data concur with our findings which suggest that stool Xpert is more useful in children with severe TB. Two

pilot studies from South Africa<sup>29,34</sup> and a multicenter study of Asian and African HIV-infected children with suspected intrathoracic TB<sup>35</sup> reported sensitivities of 41%–75%, with specificities >97.5% for stool Xpert versus culture of respiratory samples. The higher sensitivity reported compared with our results can be explained by the severe spectrum of TB in children with Xpert-positive stool included in these studies. In the study by Nicol, 100% (N = 8) of children with positive stool Xpert had alveolar consolidation; 86% had nodal airway compression.<sup>34</sup> In the study by Marcy et al,<sup>35</sup> 48% of children (14/29) with bacteriologically confirmed TB were smear positive, higher than typically observed in pediatric paucibacillary TB. In our initial pilot study (N = 14), all children with Xpert-positive stool had severe intrathoracic TB.<sup>29</sup> In our current study, stool Xpert was positive only in children with radiologically severe TB, with a sensitivity of 82% for smear-positive TB and 45% for bacteriologically confirmed cases with severe disease. This is consistent with a laboratory study where the level of detection for Xpert on Macaque stools was determined to be approximately 1000 colony forming units/mL,<sup>36</sup> whereas in respiratory samples it is approximately 100 colony forming units/mL.<sup>37</sup>

Given this higher detection threshold of Xpert on stool compared with respiratory samples, stool Xpert will only detect a proportion of children with bacteriologically confirmed TB. However, when direct comparison was made with the diagnostic potential of a single GA or IS sample, stool was not substantially inferior. Furthermore, stool Xpert had a substantial incremental yield over culture of the first respiratory sample, indicating that although stool Xpert will miss some cases, the total number detected is comparable to a single respiratory sample, which is more invasive than stool testing. Testing multiple stool samples could also improve sensitivity, though cost and feasibility would need to be considered. With more sensitive molecular tools soon to become available for evaluation, hopefully also in children,<sup>38</sup> the clinical utility of stool for TB diagnosis in children may be further improved.

An important factor to consider when applying new diagnostic tests to stool samples is the sample processing method. The different protocols used in published studies could partly explain the variable results observed. For molecular analysis, stool samples require careful processing to remove polymerase chain reaction inhibitors and particulate matter that can interfere with assay performance.<sup>36,39–41</sup> Stool processing currently remains relatively labor intensive, but work is ongoing to develop centrifugation-free protocols applicable to primary/district level healthcare facilities.<sup>36</sup> As optimized methods for processing stool for TB testing have not yet been established, parallel comparisons of the most promising sample processing procedures for stool should be evaluated in future study.

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## Chapter 7

# Evaluation of a novel centrifugation-free processing method for the detection of *Mycobacterium tuberculosis* from stool in young children using the Xpert MTB/RIF assay

### 7.1 Rationale

Following my initial pilot study demonstrating that Xpert could effectively detect *M.tb* from paediatric stool specimens, there was renewed interest among paediatric researchers to revisit stool as a potentially useful specimen to diagnose PTB in children. In 2013, the WHO endorsed Xpert for the diagnosis of TB in children<sup>232</sup>, following a systematic review showing improved sensitivity and specificity of Xpert compared to smear microscopy<sup>25</sup>. Although stool was not a validated specimen for use on the Xpert platform, there was interest in novel applications of this assay, including using non-respiratory samples. A number of studies evaluated Xpert testing of stool for the diagnosis of paediatric PTB, reporting sensitivities of 47-75% compared to culture and Xpert of respiratory specimens<sup>233-235</sup>. However, the methods for processing stool remained too complex and resource-intensive for routine care setting. The need for centrifugation in all published methods was identified as a major barrier for uptake in resource-limited settings<sup>233-235</sup>. A new,

centrifugation-free stool processing kit was developed by Banada *et al* at the Alland laboratory (Rutgers Biomedical and Health Sciences, Newark, NJ) to simplify processing of paediatric stool specimens for Xpert testing. Initial proof-of-concept work confirmed that this method was able to detect *M.tb* on paediatric stool specimens using Xpert <sup>236</sup>.

Given our group's experience in stool-based diagnosis and the available diagnostic platform, our site was approached by FIND to participate in a multi-centre study for the validation of this novel stool processing kit in a large cohort of children. This sub-study was implemented in parallel to the studies described in Chapters 6 and 8.

## **7.2 Study Aims**

The aim of this study was to evaluate a novel, centrifugation-free method for stool-based diagnosis of PTB in children, compared to diagnosis by Xpert and culture on respiratory specimens.

## **7.3 Methods**

Children <13 years of age with suspected PTB were enrolled at two referral hospitals in Johannesburg (Site 1) and Cape Town (Site 2), South Africa. At Site 1, eligibility criteria were broad and included any child whom the attending clinician decided to investigate for a clinical suspicion of PTB. At Site 2, the well-defined standard eligibility criteria are described in Chapter 4. A minimum of one and up to two stool specimens per child were collected. A minimum of one and up to four

respiratory specimens (GA/ESP or IS) were collected per child and evaluated by smear microscopy, liquid culture and Xpert. The stool processing kit was applied to two initial stool masses from each child: 1) 0.6g and 2) a stool swab. For the 0.6g protocol, the raw stool specimen was mixed with a proprietary stool buffer and briefly vortexed with glass beads to remove particulate matter. After incubation for 30 minutes, the stool suspension was transferred into the stool processing filter unit, and a plunger was attached to squeeze out the liquid (2ml) into the Xpert cartridge. Stool swabs were processed in a similar way, except that the swab was left to incubate in the buffer, which was then transferred directly into the Xpert cartridge after brief vortexing. Respiratory specimens were processed at the NHLS laboratories at both sites according to standard protocols.

## **7.4 Results**

Of 302 children initially enrolled, 280 children were included in analysis. The median age was 15.5 months, with 35 (12.5%) HIV-infected. The two clinical cohorts differed significantly, with higher HIV prevalence, malnutrition, co-morbidity and mortality seen at Site 1 in Johannesburg. Children from Site 2 in Cape Town had a significantly higher prevalence of TB exposure, TST positivity and chest radiographs typical of TB. In addition, the majority of children from Site 1 had only one respiratory specimen collected, whereas most children from Site 2 had  $\geq 3$  respiratory specimens collected.

Compared to detection by Xpert on the first respiratory specimen, both stool masses performed similarly, with sensitivity 44.4% (95% CI: 13.7-78.8%) and

specificity >99%. In 249 (88.9%) children who had a second stool tested, the combined sensitivity of two stool tests vs. the first respiratory Xpert was 70.0% (95% CI: 34.8-93.3%) for the 0.6g and 50.0% (95% CI: 18.7-81.3%) for swab, with high specificity >98%. Re-testing of stool specimens with initial non-determinate Xpert results substantially reduced non-determinate rates from 9.3% to 3.9% for 0.6g, and from 8.6% to 4.3% for swab. A single respiratory culture detected 17/23 (73.9%; 95% CI 51.6-89.8%) microbiologically confirmed cases overall, compared to 7/23 (30.4%; 95% CI 13.2-52.9%) for a single stool Xpert test using either 0.6g or swab. Stool Xpert did not detect any rifampicin resistance, returning negative or indeterminate results for all 5 children with confirmed rifampicin resistance on LPA done on positive respiratory cultures.

## 7.5 Conclusions

This stool processing method is well-suited for settings with limited capacity for respiratory specimen collection, as it was easy to complete and required no expensive or complicated equipment. This was the first study to systematically demonstrate the incremental value of a second stool test, both to improve the detection of *M.tb* and to reduce non-determinate rate. However, the diagnostic sensitivity of this method on stool was still substantially lower than the diagnostic yield of Xpert or culture on one respiratory specimen. Furthermore, due to the low bacillary concentrations in stool specimens, the utility of simultaneous rifampicin resistance detection was negated by the high rate of indeterminate rifampicin resistance results. This simple method could however still prove useful in future, if



more sensitive molecular assays could be developed for the detection of paucibacillary TB.

## 7.6 Citation

**Walters E**, Scott L, Nabeta P, Demers AM, Reubenson G, Bosch C, David A, van der Zalm M, Havumaki J, Palmer M, Hesselning AC, Ncayiyana J, Stevens W, Alland D, Denkinger C, Banada P. 2018. Molecular detection of Mycobacterium tuberculosis from stool in young children using a novel centrifugation-free processing method. J Clin Microbiol doi:10.1128/jcm.00781-18.

## 7.7 Candidate's Contribution

I was jointly responsible (with L. Scott) for the design, implementation and data collection for this study. I jointly designed and undertook the statistical analyses, interpreted the results, drafted and finalised the manuscript after feedback from the other co-authors.

## **7.8 Published Manuscript**



# Molecular Detection of *Mycobacterium tuberculosis* from Stools in Young Children by Use of a Novel Centrifugation-Free Processing Method

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**ABSTRACT** The microbiological diagnosis of tuberculosis (TB) in children is challenging, as it relies on the collection of relatively invasive specimens by trained health care workers, which is not feasible in many settings. *Mycobacterium tuberculosis* is detectable from the stools of children using molecular methods, but processing stool specimens is resource intensive. We evaluated a novel, simple, centrifugation-free processing method for stool specimens for use on the Xpert MTB/RIF assay (Xpert), using two different stool masses: 0.6 g and a swab sample. Two hundred eighty children (median age, 15.5 months; 35 [12.5%] HIV infected) with suspected intrathoracic TB were enrolled from two sites in South Africa. Compared to a single Xpert test on respiratory specimens, the sensitivity of Xpert on stools using the 0.6-g and swab samples was 44.4% (95% confidence interval [CI], 13.7 to 78.8%) for both methods, with a specificity of >99%. The combined sensitivities of two stool tests versus the first respiratory Xpert were 70.0% (95% CI, 34.8 to 93.3) and 50.0% (95% CI, 18.7 to 81.3) for the 0.6-g and swab sample, respectively. Retesting stool specimens with nondeterminate Xpert results improved nondeterminate rates from 9.3% to 3.9% and from 8.6% to 4.3% for 0.6-g and swab samples, respectively. Overall, stool Xpert detected 14/94 (14.9%) children who initiated antituberculosis treatment, while respiratory specimens detected 23/94 (24.5%). This stool processing method is well suited for settings with low capacity for respiratory specimen collection. However, the overall sensitivity to detect confirmed and clinical TB was lower than that of respiratory specimens. More sensitive rapid molecular assays are needed to improve the utility of stools for the diagnosis of intrathoracic TB in children from resource-limited settings.

**KEYWORDS** children, diagnosis, stool, tuberculosis

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E.W. and L.S. contributed equally to the manuscript.

**T**uberculosis (TB) is a leading cause of death in children globally. Data from a recent modeling study calculated that TB ranked as one of the top 5 killers of young children, with an estimated 191,000 annual deaths in children <5 years old (1). The majority (96%) of deaths occurred in children who did not receive treatment (1). The substantial challenges in confirming TB in children contribute to underdetection and lack of access to treatment.

Some of the current barriers to diagnosing pulmonary (intrathoracic) TB (PTB), particularly in young children, include difficulties in obtaining adequate specimens for microbiological confirmation (2) and the low bacterial load in pulmonary secretions. Although culture remains the gold standard for TB diagnosis, it has low sensitivity (30 to 40%) for paucibacillary pediatric TB (3, 4) and a long turnaround time, requires well-equipped laboratory infrastructure, and is prone to contamination. In some resource-constrained settings, these factors limit the feasibility of culture-based diagnosis in children. Molecular tests like the Xpert MTB/RIF assay (Xpert) (Cepheid, Sunnyvale, CA) have become widely used, even in low-resourced settings. Although the sensitivity of Xpert is lower than that of culture in children (estimated 67% for diagnosis of PTB) (5), the assay is fully automated, and the rapidity of results and need for minimal processing before testing, while also informing drug susceptibility status for rifampin, makes Xpert an attractive alternative to culture. With the development of the Xpert ultra, with improved sensitivity compared to that of the Xpert (6), the use of this technology is likely to expand further.

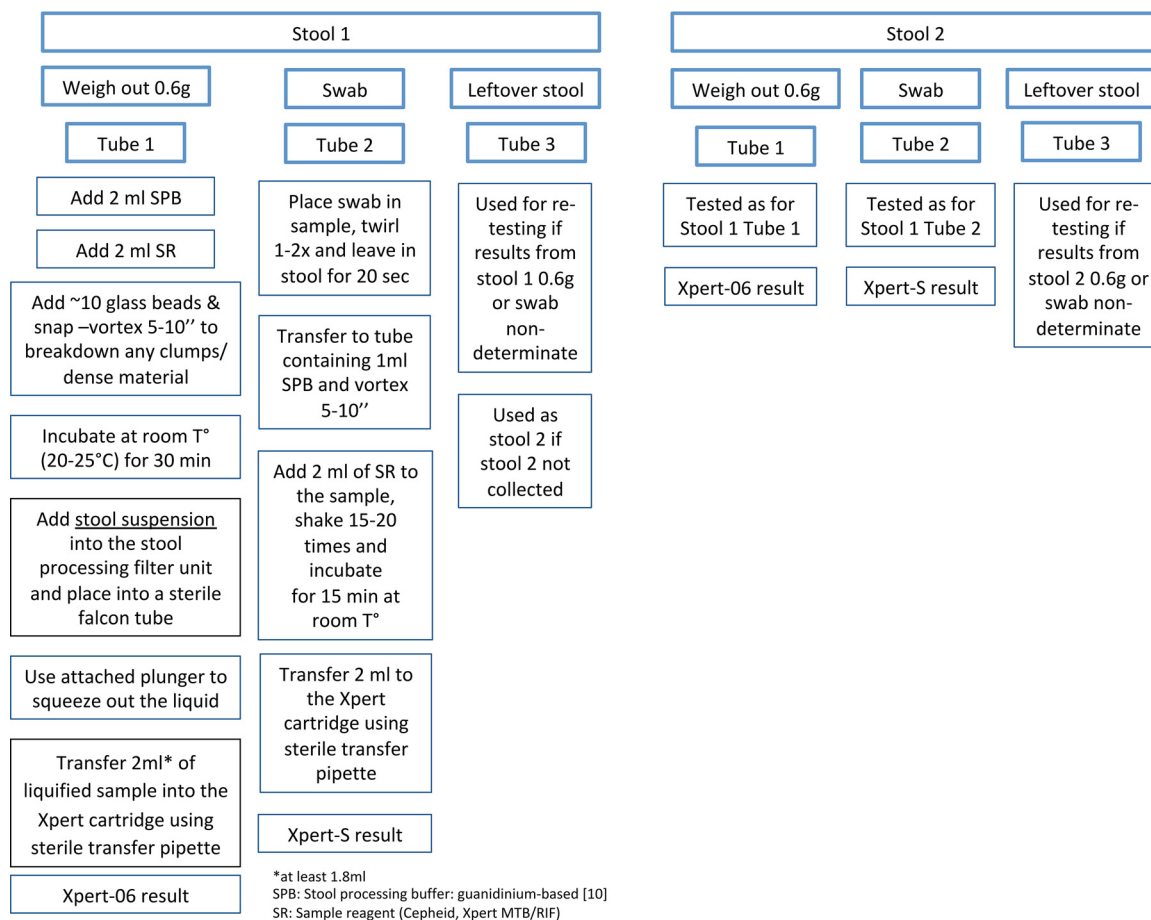
The use of alternative, less invasive specimens, including stool samples, which are potentially easier to collect than gastric aspirates (GA), induced sputum (IS), and nasopharyngeal aspirates (NPA), may encourage better attempts at microbiological confirmation of TB, particularly in young children. A number of studies have evaluated the Xpert used on stool for the diagnosis of pediatric PTB, reporting sensitivities of 47 to 75% compared to those of culture and Xpert on respiratory specimens (7–9). However, the procedure for stool processing to date has been labor intensive and time consuming, requiring multiple steps and centrifugation (7–9). A new stool processing (SP) method was developed by Banada et al. at the Alland laboratory (Rutgers Biomedical and Health Sciences, Newark, NJ), for easier, more rapid processing of pediatric stool specimens for Xpert testing (Fig. 1) (10). Initial proof-of-concept work confirmed that *Mycobacterium tuberculosis* was detectable by Xpert on pediatric stool specimens processed by this method.

In this hospital-based study, we evaluated the SP method for Xpert testing using different initial stool masses, i.e., 0.6-g and swab samples (referred to below as Xpert-06 [Xpert on 0.6 g stool] and Xpert-S [Xpert on stool swab]), respectively, for the diagnosis of PTB in children investigated at two sites in South Africa. We report the diagnostic yield of this method, the incremental value of testing a second stool sample, and the impact of pretest probability and influence of a reference standard when evaluating the diagnostic performance of this new testing method for pediatric PTB.

(This work forms part of the body of work toward a Ph.D. degree for E. Walters.)

## MATERIALS AND METHODS

**Study setting, population, and eligibility.** The study was conducted at two public referral hospitals offering general and specialized pediatric care. At site 1, the Rahima Moosa Mother and Child Hospital in Johannesburg, the study was part of routine clinical care; clinical and microbiological investigations, except for stool testing, followed local practice. All children identified by the attending medical staff as requiring investigation for suspected PTB were eligible for enrollment, including children with chronic or recurrent respiratory symptoms, severe or complicated pneumonia, severe or unexplained malnutrition, and extrapulmonary symptoms compatible with TB in conjunction with abnormal chest radiology. At site 2, Desmond Tutu TB Centre, enrolling at Tygerberg and Karl Bremer Hospitals in Cape Town, an ongoing prospective diagnostic TB study (parent study) supported this work as a substudy. The parent study had well-defined eligibility criteria, a study-specific specimen collection schedule, and protocols for mycobacterial testing and also included an evaluation of Xpert on stools, different from this substudy (7). Children were eligible to be part of both the parent and substudy; therefore, a number of children enrolled in this substudy have been described previously (7). Eligibility was based on any well-defined symptom of PTB (11) or, alternatively, a short history of cough but also other evidence suggestive of TB,



**FIG 1** Stool specimen preparation flow diagram and outline of stool processing methods.

including TB exposure in the preceding 12 months, a reactive Mantoux tuberculin skin test (TST), or chest X-ray (CXR) suggestive of TB (using a standard reading form).

Children were excluded if they had received more than one dose of antituberculosis treatment within 60 days prior to enrollment, had extrathoracic TB without concurrent suspected intrathoracic PTB, were clinically unstable, or lived remotely with no access to transport for follow-up visits.

**Study procedures.** Clinical evaluation included information on any previous TB episodes, current/recent (past 12 months) TB exposure, and TST if available. HIV testing followed local guidelines: at site 1, in children <18 months of age, the mother was tested using two rapid HIV tests from different manufacturers. If these were negative, the child was classified as “HIV unexposed” and not tested unless clinically indicated. If the two rapid HIV tests were discordant, an HIV antibody test (enzyme-linked immunosorbent assay [ELISA]) was performed. In children of HIV-infected mothers and in all children >18 months of age, a confirmatory test was completed on the child: HIV DNA PCR if <18 months old or HIV ELISA for children ≥18 months old. At site 2, all children were initially tested by either an HIV DNA PCR or ELISA as described for site 1, unless phlebotomy was insufficient, in which case a rapid HIV test was performed. This was followed by a confirmatory test if positive.

CXRs (frontal and lateral films) were completed and evaluated retrospectively by independent blinded experts, reporting according to a standardized format. Severe TB was defined as any of the following: lymph node disease with airway compression, any cavitation, miliary TB, or expansile pneumonia (12).

**Specimen collection for TB testing. (i) Respiratory specimens.** At site 1, health care workers collected an expectorated sputum (ES) or IS specimen, depending on the child’s ability to expectorate spontaneously. In addition, a GA was collected as a second respiratory specimen in a subset of children <5 years of age. Each single specimen was collected in two specimen containers: one specimen was processed onsite using Xpert; the second was sent to the nearby National Health Laboratory Service (NHLS) in Braamfontein, Johannesburg, for decontamination and concentration using *N*-acetyl-L-cysteine (NALC)-NaOH (final NaOH concentration, 1.25%) for TB microscopy and liquid culture (mycobacterial growth indicator tube [MGIT]).

At site 2, study personnel collected a minimum of two respiratory specimens on two separate occasions (4 h apart if on the same day or on two consecutive days). For children unable to expectorate (typically <5 years old), samples included a GA and an IS (with nasopharyngeal suctioning). For children

able to expectorate, an early morning ES and an IS (with expectoration) were collected. Samples were processed at NHL S Tygerberg, using NALC-NaOH (final NaOH concentration, 1.25%) (7) and tested using fluorescent smear microscopy, MGIT, and the Xpert test.

At both sites, acid-fast-bacillus (AFB)-positive cultures were identified as *M. tuberculosis* complex by using the MTBDR<sub>plus</sub> line probe assay (LPA). Second-line phenotypic drug susceptibility testing (DST) was performed at site 1 if resistance to any of the first-line drugs was reported and at site 2 if rifampin resistance was detected on LPA.

**(ii) Stool specimens.** At both sites, a minimum of one and up to two stool specimens were collected from each participant according to study standard operating procedures, no more than 48 h apart and within 7 days of collection of respiratory specimens. Stool was collected from the diaper in young children or from cling wrap fitted over the toilet seat in toilet-trained children. Infants with liquid stools had nylon waterproof material fitted under the diaper for collection of stool. Stool was transferred into a 25-ml fecal cup with the included spoon (PLPS109148; LASEC, Cape Town, South Africa). At site 1, stool specimens were tested immediately onsite in a point-of-care laboratory adjacent to the children's ward; therefore, no storage or transport was required. Specimens at site 2 were transported in a cooler box to the laboratory as soon as possible after collection (same day) and were refrigerated at 2 to 8°C until processing (within 72 h of collection). Caregivers collecting stool at home were instructed to keep the specimens refrigerated until they were collected by the study team.

Collection of a stool mass of at least 2 g (6 scoops using the spoon fitted onto the cap of the fecal cup) was recommended, to allow for the two volumes to be tested (swab and 0.6 g). Each of the two stool specimens collected was tested individually. However, if only one stool specimen was available, the same specimen could be used for the second analysis if the residual volume was sufficient. Stool specimens were tested onsite using the Xpert SP protocol (Fig. 1). The laboratory technician recorded the macroscopic appearance of each stool specimen in a standard form before testing.

**Treatment and follow-up.** The results of the Xpert test (respiratory specimens and stool) and MGIT culture, including DST, were reported to the attending clinicians. Attending clinicians decided on antituberculosis treatment according to clinical guidelines and local standard of care. These treatment decisions were documented by the study team.

A follow-up visit 8 to 10 weeks after enrollment was conducted. A study clinician with access to all laboratory results assessed the response to antituberculosis treatment based on symptoms, signs, weight gain, and CXR. In children not initiated on antituberculosis treatment, symptom resolution was assessed. Children were then classified according to international consensus clinical case definitions (13).

**Statistical analysis.** Descriptive analyses were completed using median values and interquartile ranges for continuous data with nonnormal distribution and proportions for discrete data.

The index test under evaluation, the stool Xpert, was compared to two reference standards: (i) a single Xpert and (ii) a single liquid culture, each on a respiratory specimen. The overall detection by stool Xpert for any method was compared to the clinical case definitions for diagnostic studies (13), where confirmed TB was defined as any child confirmed by Xpert or culture on any respiratory specimen: this analysis follows Standards for Reporting of Diagnostic Accuracy (STARD) guidelines (14), as the clinical case definitions incorporate the gold standard for pulmonary TB by defining "confirmed TB" as TB confirmed by Xpert or culture on any respiratory specimens. In addition, the sensitivity of testing one stool by both 0.6-g and swab sample methods was compared to the sensitivity of a single respiratory culture for the detection of (i) confirmed TB and (ii) clinical TB, defined as the clinician's decision to treat for TB.

Nondeterminate (invalid/error/no result) results for Xpert on respiratory and stool specimens were repeated on the same specimen if sufficient specimen was available. The final result used for the diagnostic analysis was the result of the second test, if done (for nondeterminate), or of the initial test, if the test was not repeated. Stool and respiratory specimens with a final nondeterminate Xpert result after repeat testing, as well as respiratory specimens with contaminated culture results, were excluded from analysis.

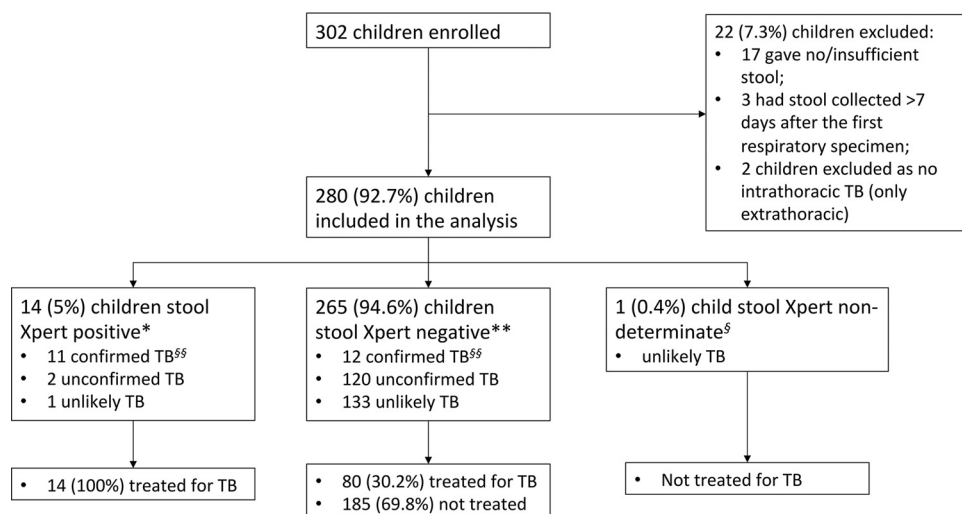
The sensitivity, specificity, and positive and negative predictive values were calculated individually for Xpert-06 and Xpert-5. The additional yield of a second stool test (using a second stool specimen if available or by retesting the first stool if the residual volume was sufficient) for each method was determined and was calculated as the percent increase in detection above that of the first stool test. The combined yield of two stool specimens (or two stool tests if one stool was used) was calculated.

Univariate analysis was used to identify factors associated with a positive stool Xpert result. As the outcome was only present in 14 children, the multivariable model only included two variables. For multivariable regression and comparative analyses, odds ratios and 95% confidence intervals (CIs) are reported, along with *P* values; a *P* value of <0.05 was considered statistically significant. Analyses were generated using Stata 14.0 special edition software (Stata Statistical Software, release 14, 2015; StataCorp LP, College Station, TX, USA). STARD guidelines were used for analysis and reporting (14).

**Ethical considerations.** The study was approved by the University of the Witwatersrand Human Research Ethics Committee (Medical) (M140251) and by the Stellenbosch University Health Research Ethics Committee (N09/11/282). Parents/legal caregivers gave written informed consent for participation in the study, and assent was obtained from children older than 7 years of age who showed adequate understanding.

## RESULTS

From December 2014 to September 2015, 302 children were enrolled; 280 (92.7%) were included in the final analysis (Fig. 2).



**FIG 2** STARD cohort flow diagram, illustrating stool culture results by consensus case definition (13).

The cohorts from sites 1 and 2 differed significantly in clinical presentation and certainty of TB disease (Table 1). Children from site 1 had a higher prevalence of severe malnutrition, perinatal HIV exposure, and HIV infection but significantly lower prevalence of TB exposure and TB infection and of radiological findings considered typical of PTB. At site 2, all but one child had  $\geq 3$  respiratory specimens collected for Xpert and TB culture, whereas  $>70\%$  of children from site 1 only had one respiratory specimen collected. Consequently, a significantly higher proportion of confirmed TB was detected at site 2 (20/132; 15.2%) than at site 1 (3/148; 2.0%).

A clinical decision was made to start antituberculosis treatment in 88 (31.4%) children at the time of enrollment and in another 6 (2.1%) during the 8 weeks following enrollment. Considering the results for all respiratory specimens collected and tested by Xpert and culture (but excluding stool Xpert as the index test under evaluation, following STARD guidelines [14]) and all available clinical data, 23 (8.2%) children overall had confirmed TB (15 by Xpert and culture, 7 only by culture, and 1 only by Xpert), 122 (43.6%) were unconfirmed for TB, and 135 (48.2%) were unlikely to have TB using international consensus definitions for diagnostic studies (13).

Follow-up was completed in 259 (92.5%) children: 15 (5.4%) were lost to follow-up and 6 (2.1%) (all from site 1) died. The deaths were from infectious causes complicated by multiple comorbid conditions (HIV, malnutrition, and cerebral palsy). Two deaths were due to TB, one with multidrug resistance.

**Diagnostic performance of one stool specimen.** Stools from 268/279 (96.1%) and 267/279 (95.7%) children tested by Xpert-06 (Xpert on 0.6 g stool) and Xpert-S (Xpert on stool swab), respectively, yielded final determinate results and were included in the analyses (see Fig. S1a and b in the supplemental material).

Case detection from stool and respiratory specimens at site 1 was low: only 3 children had confirmed TB from respiratory specimens, 2 (66.7%) of whom were also stool Xpert positive. Three additional children were stool Xpert positive but negative on all respiratory specimens. In addition, site 1 had no positive results from Xpert-S. At site 2, 20 children were microbiologically confirmed on respiratory specimens, of whom 9 (45.0%) were also stool Xpert positive. All cases detected on stool had positive results on respiratory specimens. Given the small numbers detected at site 1, diagnostic accuracy analyses from the two sites were combined. Individual site analyses are presented in Tables S1 and S2.

Compared to Xpert on the first respiratory specimen, the two stool testing methods performed similarly, with sensitivity of 44.4% (4/9; 95% CI, 13.7 to 78.8) and specificity of  $>99\%$  (Table 2). Three children had stool specimens positive on Xpert-06 and

**TABLE 1** Demographic, clinical, and bacteriological characteristics of the two study cohorts

Characteristic <sup>a</sup>	Value [no. (%) unless otherwise indicated] for:			P value
	All children (n = 280)	Children at site:		
		1 (n = 148)	2 (n = 132)	
Median mo of age (IQR)	15.5 (10.6–29.1)	15.5 (10.9–24.3)	16.6 (5.2–34.3)	0.856
Male	158 (56.4)	86 (58.1)	72 (54.6)	0.548
Ethnicity				
Mixed race	85 (30.4)	15 (10.1)	70 (53.0)	<0.001
Black African	191 (68.1)	129 (87.2)	62 (47.0)	
Indian	3 (1.1)	3 (2.0)		
Caucasian	1 (0.4)	1 (0.7)		
Perinatal HIV exposure	96 (34.3)	63 (42.8)	33 (25.0)	0.009
HIV infected	35 (12.5)	24 (16.2)	11 (8.3)	0.049
On ART at presentation	23 (65.7)	16 (66.7)	7 (63.6)	0.861
Previous antituberculosis treatment	19 (6.8)	6 (4.1)	13 (9.8)	0.054
Median WAZ (IQR)	−2.3 (−3.7 to −1.1)	−3.2 (−4.2 to −2.2)	−1.4 (−2.2 to −0.6)	<0.001
Median HAZ (IQR) (n = 277)	−1.8 (−2.9 to −0.9)	−1.8 (−3.2 to −0.9)	−1.8 (−2.8 to −0.9)	0.316
WAZ of <−2	160 (57.1)	117 (79.1)	43 (32.6)	<0.001
Evidence of BCG immunization	267 (95.4)	142 (95.9)	125 (94.7)	0.062
≥1 well-defined TB symptom	206 (73.6)	111 (75.0)	95 (72.0)	0.566
TST positive	27 (17.7) (n = 152)	2 (4.0) (n = 50)	25 (24.5) (n = 102)	0.002
Exposure to identified TB source case	100 (35.7)	17 (11.5)	73 (55.3)	<0.001
CXR typical of TB (%)	33 (12.8) (n = 258)	8 (6.2) (n = 130)	25 (19.5) (n = 128)	0.001
Investigated by Xpert/culture with:				
1 respiratory specimen	105 (37.5)	105 (70.9)	0	<0.001
2 respiratory specimens	44 (15.7)	43 (29.1)	1 (0.8)	
≥3 respiratory specimens	131 (46.8)	0	131 (99.2)	
Treated for TB <sup>b</sup> (%)	94 (33.6)	42 (28.4)	52 (39.4)	0.067
Clinical case categories				
Confirmed TB <sup>c</sup>	23 (8.2)	3 (2.0)	20 (15.2)	<0.001
Smear positive	4 (17.4)	1 (33.3)	3 (15.0)	
Xpert positive	16 (69.6)	3 (100)	13 (65.0)	
Culture positive	22 (95.7)	2 (66.6)	20 (100)	
Unconfirmed TB <sup>d</sup>	122 (43.6)	60 (40.5)	62 (47.0)	
Unlikely TB	135 (48.2)	85 (57.4)	50 (37.9)	
Follow-up status at mo 2				
Attended follow-up	259 (92.5)	128 (86.5)	131 (99.2)	<0.001
Lost to follow-up	15 (5.4)	14 (9.5)	1 (0.8)	0.001
Died	6 (2.1)	6 (4.1)	0	0.02

<sup>a</sup>IQR, interquartile range; ART, antiretroviral treatment; WAZ, weight-for-age Z score according to UK growth charts of 1990 (23); BCG, *Mycobacterium bovis* bacillus Calmette-Guérin; TST, Mantoux tuberculin skin test; CXR, chest radiograph.

<sup>b</sup>Includes children initiated on antituberculosis treatment within 2 months of enrollment.

<sup>c</sup>Includes only children confirmed by respiratory specimens. All smear-positive cases were also Xpert and culture positive; 15/16 Xpert-positive cases were also culture positive; 15/22 culture-positive cases were also Xpert positive.

<sup>d</sup>Three children with unconfirmed TB (i.e., mycobacteriology negative on respiratory specimens) were stool Xpert positive, all from site 1.

Xpert-S, while two were positive only on Xpert-06 and one only on Xpert-S. Xpert-06 detected *M. tuberculosis* in two children with negative respiratory Xpert, while Xpert-S detected one additional child with negative respiratory Xpert.

The sensitivity compared to the results for one respiratory specimen culture was lower for both methods, with sensitivity of 25.0% (4/16; 95% CI, 7.3 to 52.4) and specificity remaining >99% (Table 2). Although Xpert-06 and Xpert-S still added two and one confirmed diagnoses, respectively, respiratory culture detected 12 children who were negative on stool Xpert.

**Incremental value of a second stool test.** A second stool test was done in 249 (88.9%) children (Fig. S2a and b): in 132 (53.0%), a separate stool specimen was collected, while in 117 (47.0%), the first stool specimen was retested.



**TABLE 2** Diagnostic value of a single stool specimen tested by 0.6-g- and swab-sample protocols (per-participant analysis)<sup>a</sup>

Stool Xpert protocol	Parameter	Ratio (%; 95% CI) using indicated assay on 1st respiratory specimen as reference standard	
		Xpert	Culture
0.6 g		<i>n</i> = 259 <sup>b</sup>	<i>n</i> = 240 <sup>c</sup>
	Sensitivity	4/9 (44.4; 13.7–78.8)	4/16 (25.0; 7.3–52.4)
	Specificity	248/250 (99.2; 97.1–99.9)	222/224 (99.1; 96.8–99.9)
	NPV	248/253 (98.0; 95.4–99.4)	222/234 (94.9; 91.2–97.3)
Swab		<i>n</i> = 259 <sup>d</sup>	<i>n</i> = 236 <sup>e</sup>
	Sensitivity	4/9 (44.4; 13.7–78.8)	4/16 (25.0; 7.3–52.4)
	Specificity	249/250 (99.6; 97.8–100)	219/220 (99.5; 97.5–100)
	NPV	249/254 (98.0; 95.5–99.4)	219/231 (94.8; 91.1–97.3)

<sup>a</sup>PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval. The ratios are as follows: for sensitivity, number positive by Xpert/number positive by reference assay; for specificity, number negative by Xpert/number negative by reference assay; PPV, number positive by Xpert which were also positive by reference assay/total number positive by Xpert; NPV, number negative by Xpert which were also negative by reference assay/total number negative by Xpert.

<sup>b</sup>One child with only the swab method on stool 1, 11 children with nondeterminate stool Xpert results, and 9 with nondeterminate respiratory Xpert results were excluded.

<sup>c</sup>One child with only the swab method on stool 1, 11 children with nondeterminate stool Xpert results, 2 with no respiratory culture done, and 26 with contaminated or lost respiratory cultures were excluded.

<sup>d</sup>One child with only the 0.6-g method on stool 1, 12 children with nondeterminate stool Xpert results, and 8 with nondeterminate respiratory Xpert results were excluded.

<sup>e</sup>One child with only the 0.6-g method on stool 1, 12 children with nondeterminate stool Xpert results, 2 with no respiratory culture done, and 29 with contaminated or lost respiratory cultures were excluded.

For Xpert-06, a second stool test gave valid results in 8/11 (72.7%) specimens that were nondeterminate on the first testing (after repeat testing of stool 1 nondeterminates). Therefore, the nondeterminate rate for stool 1 and stool 2 combined was 3/279 (1.1%; 3 samples did not have a second test). The second stool test added 5 additional confirmed cases (*n* = 9 total cases detected from second stool specimen) to the 6 cases which had already been detected by stool specimen 1 (*n* = 11 total cases positive on Xpert-06; incremental detection of 83.3%).

For Xpert-S, a second stool test gave valid results in 11/12 (91.7%) specimens that were nondeterminate on the first testing. Therefore, the nondeterminate rate for stool 1 and stool 2 combined was 1/280 (0.4%; one sample did not have a second test). A second stool test added two additional confirmed cases (*n* = 5 total cases detected from stool 2), to the 5 cases already detected by stool 1 (*n* = 7 total cases positive on Xpert-S; incremental detection of 40%).

The combined sensitivity of two stool tests versus the first respiratory Xpert increased to 70.0% (95% CI, 34.8 to 93.3) for Xpert-06 and to 50.0% (95% CI, 18.7 to 81.3) for Xpert-S while retaining high specificity for both methods (Table 3). The slightly lower specificity observed for Xpert-06 was due to three cases who were positive on stool but negative on respiratory Xpert. Compared to the first respiratory culture, the sensitivity was 41.2% (95% CI, 18.4 to 67.1) for Xpert-06 and 35.3% (95% CI, 14.2 to 61.7) for Xpert-S, with a specificity of >99% for both methods (Table 3).

Comparing the clinical case definitions (13) to the combined results from stools 1 and 2, Xpert-06 was positive in 8/23 (34.8%) children with confirmed TB, 2/122 (1.6%) with unconfirmed TB, and 1/135 (0.7%) with unlikely TB. Xpert-S was positive in 7/23 (30.4%) children with confirmed TB and did not detect any unconfirmed or unlikely TB cases. Considering any positive stool Xpert result (from Xpert-06 or Xpert-S), 14 children were detected by testing on stool (Fig. 2). Three of the 14 children detected on stool Xpert had positive stool Xpert-06 but negative respiratory tests: two only had one IS collected, which was negative on smear, Xpert, and culture; one child had an IS, which was negative on all TB tests, and a GA, which was smear and Xpert negative, and the culture was contaminated. All three children were under 20 months of age and had no prior TB history. One was HIV

**TABLE 3** Combined diagnostic value of stools 1 and 2<sup>a</sup>

Stool Xpert protocol	Parameter	Ratio (%; 95% CI) using indicated assay on 1st respiratory specimen as reference standard	
		Xpert	Culture
0.6 g		<i>n</i> = 267 <sup>b</sup>	<i>n</i> = 244 <sup>c</sup>
	Sensitivity	7/10 (70.0; 34.8–93.3)	7/17 (41.2; 18.4–67.1)
	Specificity	253/257 (98.4; 96.1–99.6)	223/227 (98.2; 95.5–99.5)
	PPV	7/11 (63.6; 30.8–89.1)	7/11 (63.6; 30.8–89.1)
Swab		<i>n</i> = 270 <sup>d</sup>	<i>n</i> = 247 <sup>e</sup>
	Sensitivity	5/10 (50.0; 18.7–81.3)	6/17 (35.3; 14.2–61.7)
	Specificity	258/260 (99.2; 97.2–99.9)	229/230 (99.6; 97.6–100)
	PPV	5/7 (71.4; 29.0–96.3)	6/7 (85.7; 42.1–99.6)
	NPV	258/263 (98.1; 95.6–99.4)	229/240 (95.2; 91.6–97.6)

<sup>a</sup>PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval. The ratios are as follows: for sensitivity, number positive by stool 1 or 2 Xpert/number positive by reference assay; for specificity, number negative by stool 1 and 2 Xpert/number negative by reference assay; PPV, number positive by stool 1 or 2 Xpert which were also positive by reference assay/total number positive by stool 1 or 2 Xpert; NPV, number negative by stool 1 and 2 Xpert which were also negative by reference assay/total no. negative by stool 1 and 2 Xpert.

<sup>b</sup>One child with only swab method on stool 1, 3 children with nondeterminate stool Xpert results and 9 with nondeterminate respiratory Xpert results were excluded.

<sup>c</sup>One child with only swab method for stool 1, 3 children with nondeterminate stool Xpert results, 3 with no respiratory culture done, and 29 with contaminated or lost respiratory cultures were excluded.

<sup>d</sup>One child with nondeterminate stool Xpert results and 9 with nondeterminate respiratory Xpert results were excluded.

<sup>e</sup>One child with nondeterminate stool Xpert results, 3 with no respiratory culture done, and 29 with contaminated or lost respiratory cultures were excluded.

infected with an acute presentation. The child had started antiretroviral therapy 2 weeks before enrollment and was critically ill with multiorgan dysfunction, multilobar pneumonia, and confirmed nosocomial sepsis. He had no known TB exposure. The child died before follow-up was completed. The other two were HIV negative, with symptoms and chest radiographs (CXR) suggestive of TB and with a good clinical response to antituberculosis treatment at the 8-week follow-up.

Using confirmed TB as the reference standard, the sensitivity of testing a single stool by both Xpert-06 and Xpert-S was 7/23 (30.4%; 95% CI 13.2 to 52.9%), versus 17/23 (73.9%; 95% CI 51.6 to 89.8%) for one respiratory culture. Using clinical TB (decision to treat for TB) as the reference, a single stool tested by Xpert-06 and Xpert-S had a sensitivity of 8/94 (8.51%; 95% CI 3.75 to 16.1%), versus 17/94 (18.1%; 95% CI 10.9 to 27.4%) for one respiratory culture.

All stool Xpert results reported low or very low semiquantitative values: of 15 positive Xpert-06 results (from 11 children), 6 (40.0%) were low and 9 (60.0%) very low; of 10 positive Xpert-S results (from 7 children), 7 (70%) were low and 3 (30%) very low.

Stool Xpert did not detect any rifampin resistance. In three of five children with rifampin resistance detected in respiratory specimens, the stool Xpert results were negative. In the other two children, Xpert-06 was rifampin indeterminate due to low bacillary loads and prolonged cycle threshold values (Table S3). Overall, 6/25 (24%) Xpert-positive stool specimens (from 5 children) gave indeterminate rifampin resistance results: 5/15 (33.3%) and 1/10 (10%) on Xpert-06 and Xpert-S, respectively. All six indeterminate results had “very low” semiquantitative values (Table S4). By comparison, none of the Xpert-positive respiratory specimens had indeterminate rifampin resistance.

**Factors associated with stool Xpert positivity.** On univariate analysis, factors associated with stool Xpert positivity were radiologically severe TB ( $P < 0.001$ ), female sex ( $P = 0.03$ ), and positive sputum smear status ( $P < 0.001$ ). On multivariable analysis, only radiologically severe TB remained strongly associated with stool Xpert positivity. Smear status could not be included in the model as it predicted stool Xpert positivity perfectly (Table 4).

**TABLE 4** Regression analysis exploring factors associated with stool Xpert positivity<sup>a</sup>

Variable	OR <sup>b</sup>	95% CI <sup>c</sup>	P value	aOR <sup>d</sup>	95% CI	P value
Sex						
Male	Reference					
Female	3.4	1.1–11.2	0.03	3.0	0.8–10.7	0.09
Age in mo	1.0	0.99–1.0	0.30			
HIV status						
Negative	Reference					
Positive	1.2	0.3–5.5	0.84			
Stool consistency						
Liquid	Reference					
Not liquid	0.90	0.5–1.6	0.73			
Stool collection time in relation to respiratory specimen collection						
After	Reference					
Same day/before	0.9	0.3–2.6	0.80			
Stool collection time in relation to TB treatment initiation						
After	Reference					
Same day/before	2.3	0.7–7.4	0.15			
TB disease severity						
Not severe	Reference					
Severe	22.1	6.5–75.4	<0.001	20.9	6.0–72.0	<0.001

<sup>a</sup>Any stool Xpert test positive; per-participant analysis.

<sup>b</sup>OR, odds ratio. "Reference" refers to the base or reference category used for the regression analyses.

<sup>c</sup>CI, confidence interval.

<sup>d</sup>aOR, adjusted odds ratio.

Although stool consistency was not associated with Xpert positivity, no positive stool Xpert results were obtained from liquid stools and only two were from solid stools (Table S5). None of the 14 stools with visible mucus were Xpert positive. One of four bloody stools was Xpert positive.

## DISCUSSION

This is the first large study, since initial proof-of-concept (10), to evaluate the performance of a novel centrifugation-free processing method for stool specimens, to assess its use with the Xpert MTB/RIF assay on stool to diagnose TB in children. Compared to microbiological confirmation using respiratory specimens, this method demonstrated diagnostic accuracy similar to those of recently published studies in young children (7, 8, 15), while studies enrolling mainly older children reported higher sensitivities for stool Xpert (9, 16, 17). This is most likely due to the lower bacillary concentrations present in respiratory specimens and, hence, in stools of young children, who seldom present with adult-type (cavitating) TB (18, 19). We have also previously found that a more severe spectrum of TB disease as evaluated by CXR (associated with higher mycobacterial load) was strongly predictive of stool Xpert positivity using a different stool processing protocol (7) in children enrolled in the parent study from Cape Town, age.

The aim of this study was to develop and evaluate a simple method to process stool, a noninvasive specimen, for use with the Xpert assay in children. This processing method is better suited to underresourced settings, as it does not require centrifugation. We showed that a simple swab gave results similar to those of the 0.6-g-sample method, although due to the small number of positive results, the comparison was not adequately powered to show equivalence. Stool swabs have previously been used for Xpert testing; however, a centrifugation-dependent method was used, which also required the stool mass collected on the swab to be weighed (8). Our method is more feasible for clinical and laboratory settings with minimal infrastructure, and as this study illustrates, the process could be performed at a point-of-care site situated close to the patient's ward. Conversely, although not statistically significant, the higher initial stool

sample volume (0.6 g) did result in the detection of *M. tuberculosis* in seven additional children compared to swab samples, which added three diagnoses (Fig. 2), indicating that further improvements using a larger stool volume and multiple samplings could improve sensitivity.

Our study also shows that the addition of a second stool test, either from a separate stool specimen or from retesting the same stool, had a substantial benefit for the diagnostic yield and for the rate of nondeterminate results. Of the initial nondeterminate results, none were due to pressure aborts, and 2 of the error results were instrument related. The remaining nondeterminate results are likely to have been caused by inhibition. Given the success of repeat testing using residual raw stool, it is unlikely that specimen processing could have been the cause of the nondeterminate results. In this study, whether a second stool was tested or the first stool was retested, the initial starting material was raw stool (not stool stored in buffer). The first step of the stool processing protocol involved adding the stool processing buffer and gently vortexing to homogenize the sample (Fig. 1). This protocol, including standing times, was followed in a standard way for all specimens. The improvement in nondeterminate results with second testing is probably explained by the inhomogeneous property of stool, resulting in PCR inhibitors and particulate matter not being completely homogenized with rapid vortexing. Experience from the Rutgers laboratory confirms that discrepant results from repeat testing of the same stool specimens frequently occurs. It is also important to note that initial valid stool results were not repeated—it is possible that if all stool specimens were tested twice, a similar proportion of second tests would have yielded nondeterminate results as for the first tests. The incremental yield of a second stool test is likely explained by the paucibacillary nature of pediatric tuberculosis, where, as also with respiratory specimens, increasing the number of specimens/tests increases diagnostic yield (10). Although additional testing results in higher costs, restricting additional tests to nondeterminate or negative Xpert results should be considered in cases with high pretest probability of disease or where confirmation of TB is most important, such as in infants and HIV-infected children or those with exposure to a drug-resistant source case.

We observed a high proportion of indeterminate rifampin results in stools, in line with very low Xpert semiquantification. It is known that in paucibacillary specimens, the rifampin resistance results in G4 Xpert cartridges may not be reliable (20, 21). Although this could potentially limit the utility of Xpert to detect rifampin-resistant TB from stool, a more sensitive assay, such as the Xpert ultra, may be able to overcome this limitation (22).

Ours is the first study to explore stool consistency in relation to diagnostic yield by the Xpert assay. Although detection of *M. tuberculosis* was not associated with stool consistency, it is interesting to note that no liquid stools generated a positive Xpert result. Of 46 liquid stool specimens, 42 were collected in ordinary diapers (2 of the remainder were collected in urine bags and 2 in the potty), which may have resulted in a large part of the stool being soaked up into the diaper. Other explanations may include inhibitors in diarrheal stools, as well as higher dilution with lower concentration of *M. tuberculosis* DNA, or possibly, none of the children with liquid stools had active TB.

A limitation of our study was the enrollment of two substantially different cohorts of children. At site 1, TB testing was part of a package of investigations for very ill children with a high burden of comorbid diseases, and Xpert testing was performed onsite directly on the raw sputum specimen. Site 2 had more strictly defined entry criteria, the pretest probability of TB disease was higher, and the Xpert test was performed in a laboratory setting on the concentrated pellet. In addition, site 2 collected more respiratory specimens for TB investigation, which resulted in a higher proportion of children confirmed by both respiratory specimens and stool. We attempted to address these differences by comparing stool Xpert results to a reference standard that was common to both groups: a single respiratory specimen tested by Xpert and culture. However, overall, site 2 contributed the majority of positive test results and drove the results for the sensitivity analyses. The small number of

confirmed cases in this study overall also resulted in wide confidence intervals around all the estimates.

Three children from site 1 had positive stool Xpert but negative respiratory mycobacteriology. Although in at least two of the three children, the stool Xpert results were likely true positives, it remains important to optimize the reference standard in order to adequately evaluate new diagnostic tests for pediatric TB. In children, collecting a minimum of two high-quality respiratory specimens, ensuring appropriate specimen storage and transport, and optimizing laboratory processes (such as specimen concentration before testing) are critical.

Our results demonstrate the superior value of respiratory specimens for the diagnosis of intrathoracic TB in children: a single respiratory culture detected more than double ( $n = 17$ ) the number of children detected by a single stool ( $n = 8$ ). These results, therefore, support efforts to promote and strengthen the capacity for collection and testing of respiratory specimens in children for microbiological investigation of TB, as stool testing remains inadequately sensitive and largely limited to the detection of severe forms of TB. In settings where the use of empirical treatment based on clinical algorithms is high, stool-based diagnosis has limited value. However, in settings where children with TB present with advanced disease and where confirmation is required to access treatment but resources are scarce, the use of stools may improve case detection.

**Conclusions.** Despite the encouraging performance of our simple, centrifugation-free stool-processing method and the value shown in testing a second stool specimen, our study reinforces that stools cannot yet replace respiratory specimens for detection of *M. tuberculosis* in children. Children with nonsevere PTB are less likely to be detected with stool Xpert, limiting the utility of this diagnostic modality primarily to children with severe disease. The diagnostic yield of a single respiratory culture was considerably superior to that of stool Xpert, allowing for completion of full drug susceptibility testing (DST). Culture of respiratory specimens remains the most sensitive diagnostic strategy for pediatric TB if resources are available. A major benefit of Xpert, however, remains the rapid turnaround time and ability to screen for rifampin resistance. In settings where children present with severe disease and where the capacity for respiratory specimen collection is limited, more sensitive rapid assays, such as Xpert ultra, combined with an easy-to-use SP kit could prove even more useful and should be urgently evaluated.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JCM.00781-18>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.7 MB.

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## Chapter 8

# Stool culture for the diagnosis of pulmonary (intrathoracic) TB in children

### 8.1 Rationale

Despite the increasing global uptake of molecular tests, particularly Xpert and more novel tools emerging more recently (Xpert MTB/RIF Ultra; Xpert Ultra, Cepheid), for the diagnosis of PTB in adults and children, mycobacterial culture remains the gold standard for diagnosis and for determination of drug susceptibility. For paediatric paucibacillary TB, culture is more sensitive than Xpert, which is negative in >30% of culture-confirmed cases <sup>25</sup>. Traditionally, stool culture is not recommended for the microbiological confirmation of *M.tb* <sup>237</sup>, due to the high potential for contamination, but published data supporting these recommendations are scarce. At the time of my study, data in children were even more limited, with only three previous paediatric studies evaluating culture of *M.tb* from stool specimens in children <sup>107,114,115</sup>, and reporting sensitivities of 15-20% compared to culture of two GA. Donald *et al* used the BACTEC radiometric culture <sup>115</sup>, while Oberhelman *et al* used both the LJ and MODS culture methods <sup>107,114</sup>. Given the difficulty in achieving microbiological confirmation of PTB in children, partly due to the challenges in obtaining respiratory specimens, and the lack of data on the utility of stool culture using the sensitive semi-automated MGIT system, I evaluated MGIT culture as a



diagnostic modality on a sub-set of children enrolled in the parent cohort study (Chapters 4 and 5).

## **8.2 Aims**

The aim of the study was to evaluate the sensitivity and specificity of stool culture to detect *M.tb* compared to culture and Xpert of up to two respiratory specimens collected from children with suspected PTB.

## **8.3 Methods**

Children enrolled in the parent study who had one stool specimen collected at enrolment were included in this sub-study. Stool specimens were homogenized with phosphate buffered saline pH 6.8 (PBS) by vortexing, and then processed with NALC-NaOH 1.25%. Following concentration at 3,000 g for 15 minutes, the pellet was resuspended in 2mL PBS: a drop was used for fluorescent Auramine-O smear microscopy<sup>128</sup>, 0.5mL was used for MGIT culture<sup>181</sup> and 1.0 mL was mixed with 2.0 mL of Xpert sample reagent, and tested by the Xpert assay (for stool processing method 1, refer to Chapter 7). Respiratory specimens were processed and tested by smear microscopy, MGIT culture and Xpert following standard NHLS protocols in the TBH NHLS laboratory.

## **8.4 Results**

Overall, 188 children were included in the study: 38 (20.2%) had “confirmed TB”, 92 (48.9%) “unconfirmed TB” and 58 (30.9%) “unlikely TB” according to the NIH

consensus case definitions<sup>174</sup>. Stool cultures were more likely to be contaminated than GA: 78/188 (41.5%) stool vs. 31/419 (7.4%) GA cultures, while contamination was found in 24/425 (5.7%) IS and 0/6 (0.0%) ESP specimens. Of the 110 children with evaluable stool culture results, stool culture detected 7/38 (18.4%) children with confirmed TB. The sensitivity and specificity of stool culture vs. culture and Xpert of two respiratory specimens were 33.3% (95% CI:11.8, 61.6) and 97.8 (95% CI: 92.4, 99.7), respectively. Stool was smear positive in 5/38 (13.2%) children with confirmed TB: all 5 were also smear, Xpert and culture positive on respiratory specimens, while 3/ 5 stool smear-positive specimens were stool culture-negative. Stool culture was negative in all cases (n=4) with DR-TB detected on respiratory specimens.

Although, overall, 26/90 (28.9%) stool specimens were collected after a median of 2 days of initiation of anti-tuberculosis therapy, there was no difference in culture positivity for stool specimens collected before (4/64; 6.3%) or after (3/26; 11.5%) initiation of antituberculosis treatment (p=0.407).

## 8.5 Conclusions

The high proportion of contaminated stool cultures precluded an accurate assessment of the sensitivity and specificity of stool culture to diagnose PTB in children. More work should be done to develop laboratory processing methods able to reduce stool contamination. The use of chlorhexidine decontamination combined with agar plate culture medium has shown promising results compared to NALC/NaOH with MGIT culture in laboratory studies, with improved recovery of *M.tb*

(19% vs 9%) and reduced contamination rates (9% vs 17%)<sup>238</sup>. However, there are no published clinical studies comparing stool vs. GA or other paediatric respiratory specimens for the diagnosis of PTB in children using the chlorhexidine method. Current available data do not support stool as an alternative to respiratory specimens for routine mycobacterial culture in children.

## 8.6 Citations

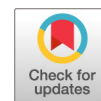
**Walters E**, Demers AM, van der Zalm MM, Whitelaw A, Palmer M, Bosch C, Draper HR, Gie RP, Hesselning AC. 2017. Stool Culture for Diagnosis of Pulmonary Tuberculosis in Children. *J Clin Microbiol* 55:3355-3365.

**Walters E**, Demers AM, van der Zalm MM, Whitelaw A, Palmer M, Bosch C, Draper HR, Gie RP, Hesselning AC. 2018. Reply to Drancourt, "Culturing Stools To Detect *Mycobacterium tuberculosis*". *J Clin Microbiol* 56. DOI 10.1128/JCM.00056-18.

## 8.7 Candidate's Contribution

I was responsible for the design, implementation and data collection for this study. I designed the statistical analyses, interpreted the results, drafted and finalised the manuscript after feedback from the other co-authors.

## **8.8 Published Manuscript**



# Stool Culture for Diagnosis of Pulmonary Tuberculosis in Children

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**ABSTRACT** Bacteriological confirmation of *Mycobacterium tuberculosis* is achieved in the minority of young children with tuberculosis (TB), since specimen collection is resource intensive and respiratory secretions are mostly paucibacillary, leading to limited sensitivity of available diagnostic tests. Although molecular tests are increasingly available globally, mycobacterial culture remains the gold standard for diagnosis and determination of drug susceptibility and is more sensitive than molecular methods for paucibacillary TB. We evaluated stool culture as an alternative to respiratory specimens for the diagnosis of suspected intrathoracic TB in a subgroup of 188 children (median age, 14.4 months; 15.4% HIV infected) enrolled in a TB diagnostic study at two local hospitals in Cape Town, South Africa. One stool culture was compared to overall bacteriological confirmation by stool Xpert and by Xpert and culture of multiple respiratory specimens. After decontamination/digestion with NALC (*N*-acetyl-L-cysteine)-NaOH (1.25%), concentrated fluorescent smear microscopy, Xpert MTB/RIF, and liquid culture were completed for all specimens. Culture contamination of stool specimens was high at 41.5%. Seven of 90 (7.8%) children initiating TB treatment were stool culture positive for *M. tuberculosis*. Excluding contaminated cultures, the sensitivity of stool culture versus confirmed TB was 6/25 (24.0%; 95% confidence interval [CI] = 9.4 to 45.1%). In addition, stool culture detected TB in 1/93 (1.1%) children with “unconfirmed TB.” Testing the same stool by Xpert increased sensitivity to 33.3% (95% CI = 18.0 to 51.8%). In conclusion, stool culture had low sensitivity for *M. tuberculosis* detection in children with intrathoracic TB. Reducing culture contamination through improved laboratory protocols may enable more reliable estimates of its diagnostic utility.

**KEYWORDS** childhood tuberculosis, stool culture, diagnosis

Although rapid molecular methods are increasingly being adopted globally for the diagnosis of tuberculosis (TB) (1), culture-based methods remain the reference (gold) standard for the diagnosis of TB and for drug susceptibility testing (DST) (1). In paucibacillary forms of TB, including most forms of TB in children and sputum-scarce or smear-negative adults with HIV-associated pulmonary TB (PTB), detection by culture is considerably superior to molecular assays. For example, the Xpert MTB/RIF assay (Xpert; Cepheid, Sunnydale, CA) has 62 to 66% sensitivity compared to culture for the diagnosis of pediatric PTB (2), and 68% for smear-negative PTB in adults (3). However, even culture in these patient groups confirms <50% of cases (4–6), partly due to low bacterial burden and the difficulty in obtaining high-quality sputum specimens (6, 7).

TB in children, although under-reported, contributes at least 10% of the disease burden globally (1), and up to 21% (8) of the total TB case load in high-burden TB settings. Children generally have good TB treatment outcomes given timely diagnosis and treatment; however, immunological immaturity, especially in young and HIV-

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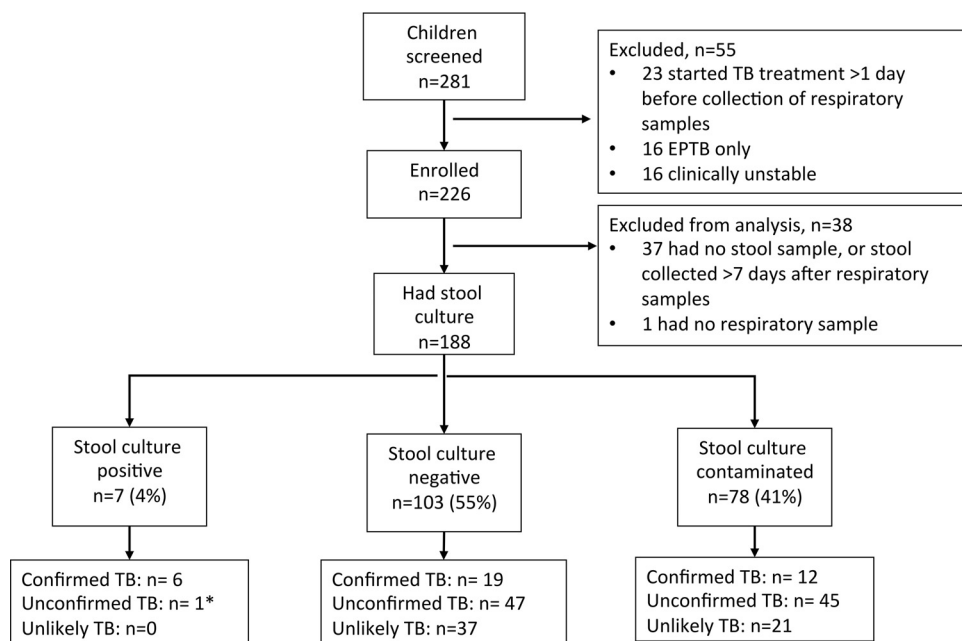
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**FIG 1** STARD cohort flow diagram, illustrating stool culture results by consensus case definition (27). EPTB, extrapulmonary tuberculosis. \*, One child was positive on stool culture only; all respiratory cultures and Xpert and stool Xpert were negative.

infected children, favors rapid progression of TB disease if the diagnosis is missed or delayed (9, 10).

In addition to the importance of confirming a diagnosis, especially in children from high-risk groups, the increasing incidence of drug-resistant TB globally (1) calls for greater efforts to pursue bacteriological confirmation in all patients at risk of drug resistance, including children, in order to treat all patients effectively and to prevent the emergence and transmission of resistant *M. tuberculosis* strains (11, 12).

Alternative methods to collect respiratory specimens, such as sputum induction, gastric aspiration, and bronchoalveolar lavage from young children, are resource intensive and relatively invasive. When collected, these specimens typically have a low mycobacterial burden, resulting in modest detection by current available tests, including mycobacterial culture and molecular assays (2, 5, 13). Collecting multiple specimens improves detection yield (14–16) but is costly and typically has to occur over consecutive days, which limits its feasibility.

Stool is easily collected from children and can be used for the detection of *M. tuberculosis* present in swallowed sputum, using both culture and molecular methods (17–26). We have recently shown that stool Xpert detects approximately one in four children with radiologically severe PTB (25). Although Xpert gives rapid results, including rifampin resistance, current diagnostic algorithms still require a cultured isolate for further DST to isoniazid and other drugs. We evaluated stool culture as a noninvasive strategy for the diagnosis of intrathoracic (pulmonary) TB in a subset of children with suspected PTB, whose stool Xpert results were reported previously (25).

## RESULTS

Cohort recruitment and characteristics are summarized in Fig. 1 and Table 1. Overall, 188 children were included in the study. Thirty-seven (19.7%) children were classified as “confirmed TB” (excluding stool culture results as the index test), 93 (49.5%) were classified as “unconfirmed TB,” and 58 (30.9%) were classified as “unlikely TB” according to international consensus clinical case definitions for intrathoracic TB in children (27). Overall, 90 (47.9%) children were treated for TB by the clinical care team. Twenty-six of 90 (28.9%) children who initiated antituberculosis therapy had stool specimens collected after treatment initiation (median, 2 days; interquartile range, 1 to 4 days).

**TABLE 1** Cohort characteristics overall and grouped by international consensus diagnostic category (27) in children presenting with suspected tuberculosis ( $n = 188$ )

Characteristic	Diagnosis <sup>a</sup>			
	All children	Children with confirmed TB <sup>b</sup>	Children with unconfirmed TB	Children with unlikely TB
Total	188 (100)	37 (19.7)	93 (49.5)	58 (30.9)
Age (mo)	14.4	17.5	15.5	12.5
Median (IQR)	7.2–25.6	8.9–28.4	9.1–26.1	5.6–20.2
Male	95 (50.0)	15 (40.5)	48 (51.6)	32 (55.2)
HIV infected	29 (15.4)	2 (5.4)	19 (20.4)	8 (13.8)
On ART at presentation	9 (31.0)	0 (0)	8 (42.1)	1 (12.5)
WAZ < -2	97 (51.6)	19 (51.4)	59 (63.4)	19 (32.8)
With evidence of BCG immunization	182 (96.8)	32 (86.5)	93 (100)	57 (98.3)
≥1 well-defined TB symptom(s) <sup>c</sup>	149 (79.3)	33 (89.2)	81 (87.1)	35 (60.3)
TST positive	48 (29.4)	23 (74.2)	21 (25.6)	4 (8.0)
<i>n</i>	163	31	82	50
Exposure to identified TB source case	105 (55.9)	25 (67.6)	71 (76.3)	9 (15.5)
CXR suggestive of TB	58 (31.9)	26 (74.3)	28 (30.1)	4 (7.4)
<i>n</i>	182	35	93	54
Treated for TB	90 (47.9)	37 (100)	38 (40.9)	15 (25.9)

<sup>a</sup>Values are expressed as number (%) unless otherwise noted in column 1. IQR, interquartile range; ART, antiretroviral therapy; WAZ, weight-for-age Z-score according to UK growth charts 1990 (44); BCG, bacillus Calmette-Guérin; TST, tuberculin skin test; CXR, chest radiograph.

<sup>b</sup>This value includes all children with positive Xpert or culture of *M. tuberculosis* from respiratory specimens or a positive Xpert result for stool. Two children were confirmed only on stool Xpert. One child whose only *M. tuberculosis*-positive test was stool culture is classified as “unconfirmed TB,” since stool culture was the index test.

<sup>c</sup>As reported previously (45).

Of the 37 children with bacteriologically confirmed TB, 28 were confirmed on respiratory specimen culture and 9 were confirmed by Xpert only (7 on respiratory specimens and two on stool; all 9 were culture negative). Stool culture was positive in 6/37 (16.2%) children with “confirmed TB,” in 1/93 (1.1%) children with “unconfirmed TB” and in none of the children with “unlikely TB.”

In order to present a fair comparison between stool and respiratory specimens, we also compared stool culture to culture of respiratory specimens in children who had stool and respiratory specimens collected on the same day ( $n = 153$ ). Stool culture sensitivity was 33.3% (95% confidence interval [CI] = 11.8 to 61.6%), with other measures of diagnostic accuracy being similar to the comparison between stool and 2 gastric aspirate (GA) or 2 sputum (SPT) specimens. The diagnostic accuracy analyses for stool culture are shown in Table 2 and detailed microbiology of stool specimens in Table 3.

If contaminated stool cultures are excluded, there was no difference in culture positivity for stool specimens collected before (4/35; 11.4%) or after (3/18; 16.7%) initiation of TB treatment ( $P = 0.667$ ). Of the children with positive respiratory cultures ( $n = 28$ ), 21 (75%) had stool collected within 1 day of the first culture-positive respiratory specimen: 12/21 (57.1%) on the same day or 1 day before the respiratory specimen and 9/21 (42.9%) 1 day after the respiratory specimen. Stool cultures were positive in 6 of these 21 (28.6%) children; 10/21 (47.6%) stool cultures were negative, and 5/21 (23.8%) were contaminated. In comparison, none of the 7 stools collected >1 day after the first culture-positive respiratory specimen were culture positive.

The 7 isolates from positive stool cultures were all drug susceptible. Drug-resistant TB was detected from respiratory specimens in 4/37 (10.8%) children with confirmed TB: 2 were at least isoniazid monoresistant, and 2 were multidrug resistant (MDR), with susceptibility to ofloxacin and amikacin confirmed on phenotypic DST. Stool culture was negative in all 4, despite stool being collected before treatment in all cases.

**TABLE 2** Diagnostic accuracy of stool culture compared to defined reference standards<sup>a</sup>

Parameter	Comparison			
	SC vs culture or Xpert <sup>b</sup>	SC vs clinical decision to treat <sup>c</sup>	SC vs culture of GA/SPT <sup>d</sup>	SC vs culture of respiratory specimens <sup>e</sup>
Stool culture result (no. of samples [+/-/total])				
Stool culture (+)	6/1/7	7/0/7	5/2/7	5/1/6
Stool culture (-)	15/88/103	46/57/103	10/91/101	10/73/83
Totals	21/89/110	53/57/110	15/93/108	15/74/89
% sensitivity or specificity (95% CI)				
Sensitivity	28.6 (11.3, 52.2)	13.2 (5.5, 25.3)	33.3 (11.8, 61.6)	33.3 (11.8, 61.6)
Specificity	98.9 (93.9, 100.0)	100.0 (93.7, 100.0)	97.8 (92.4, 99.7)	98.6 (92.7, 100.0)
PPV or NPV (95% CI)				
PPV	85.7 (42.1, 99.6)	100.0 (59.0, 100.0)	71.4 (29.0, 96.3)	83.3 (35.9, 99.6)
NPV	85.4 (77.1, 91.6)	55.3 (45.2, 65.1)	90.1 (82.5, 95.1)	88.0 (79.0–94.1)

<sup>a</sup>GA, gastric aspirate; SPT, sputum; IS, induced sputum; (+), positive; (-), negative; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

<sup>b</sup>That is, stool culture compared to culture or Xpert of up to 2 GA/SPT and 2 IS specimens; *n* = 110 (78 children with contaminated stool culture were excluded).

<sup>c</sup>That is, stool culture compared to clinical decision to treat; *n* = 110 (78 children with contaminated stool culture were excluded).

<sup>d</sup>That is, stool culture compared to culture of 2 GA/SPT specimens; *n* = 108 (78 children with contaminated stool culture and 2 with contaminated GA cultures were excluded).

<sup>e</sup>That is, stool culture compared to culture of respiratory specimens collected on the same day; *n* = 89 (64 children with contaminated stool culture and 2 with contaminated respiratory cultures were excluded).

Two of the seven (28.6%) culture-positive and 3/103 (2.9%) culture-negative stool specimens were smear positive (Table 3). The three culture-negative, smear-positive stool specimens were all collected before starting TB treatment, and all were processed between 12 and 72 h after collection. All smear-positive stool samples originated from children who had confirmed TB: respiratory specimens from these children were all smear, Xpert, and culture positive. No nontuberculous mycobacteria were isolated from stool.

Culture was contaminated in 78/188 (41.5%) stool versus 31/419 (7.4%) GA, 24/425 (5.7%) induced sputum (IS), and 0/6 (0.0%) SPT specimens. Although not subjected to microbiological identification, contaminating organisms were compatible with fungal and bacterial overgrowth.

**TABLE 3** Stool microbiology results grouped by culture, Xpert MTB/RIF, and smear results<sup>a</sup>

Culture	TTP (days)	Xpert	Xpert semiquantitative	Smear	Smear grade	No. of stool specimens <sup>b</sup> ( <i>n</i> = 188)
Pos MTB	9	Det	VL	Pos	1+	1
	16	Det	L	Neg		1
	25	Det	L	Neg		1
	26	Det	L	Neg		1
	19	Neg		Neg		1
	22	Neg		Neg		1
	12	E/I/NR		Pos	1+	1
	Neg		Det	M	Pos	3+
		Det	VL	Pos	2+	1
		Det	L	Pos	Scanty	1
		Det	VL	Neg		1
		Det	VL	Neg		1
		Det	L	Neg		1
		Neg		Neg		85
		E/I/NR		Neg		12
Contaminated		Det	VL	Neg		1
		Neg		Neg		69
		E/I/NR		Neg		8

<sup>a</sup>TTP, time to positivity; Pos, positive; Det, *M. tuberculosis* detected; VL, very low; L, low; Neg, negative; E/I/NR, error, invalid, or no result; M, medium.

<sup>b</sup>One stool specimen per participant (*n* = 188 participants).



**Incremental value of Xpert on stool for *M. tuberculosis* detection.** Of the seven stool specimens with positive cultures, the Xpert result on the same specimens was positive in four (57.1%). However, the incremental value of testing all stool specimens with Xpert as well was 100%, since Xpert detected *M. tuberculosis* in an additional seven stool specimens, six of which were stool culture negative and one had a contaminated culture (Table 3). The sensitivity/specificity of combined stool culture and Xpert versus confirmed TB based on respiratory specimens alone was 33.3% (95% CI = 18.0 to 51.8%)/98.0% (95% CI = 94.2 to 99.6%), respectively, if including participants with either a valid stool Xpert or stool culture result ( $n = 180$ ). Eight children who had both stool Xpert invalid and stool culture contaminated were excluded from this calculation. The reduced specificity is due to three children who were positive on stool Xpert or stool culture alone, but negative on all respiratory specimens. None of the children with MDR-TB were stool Xpert positive.

**Spectrum of TB disease in children with positive stool culture.** Six of the seven children with positive stool cultures showed severe disease on chest radiographs (CXR) (28), including four with cavities. One child had a normal CXR, and all cultures except stool were negative. The child was started on antituberculosis therapy clinically before stool culture results were available, based on suggestive symptoms, a positive tuberculin skin test (TST), and significant TB exposure history (mother with TB), and responded well to TB treatment. None of the seven had extrathoracic TB.

**Stool culture in abdominal TB.** Of seven children with a clinical/sonographic diagnosis of abdominal TB, four had negative and three had contaminated stool cultures. For one child, *M. tuberculosis* was detected by Xpert in a stool sample.

## DISCUSSION

The ability to culture *M. tuberculosis* from an appropriate clinical specimen allows for characterization of the mycobacterial isolate, including genotyping and DST, and remains a critical part of clinical management of TB in children and adults. We have already shown that in children with severe intrathoracic TB, stool Xpert can provide a rapid confirmation in a substantial proportion of children and can directly inform clinical care (25). Since culture is more sensitive than Xpert, especially in paucibacillary TB (2, 29), we evaluated the diagnostic utility of stool culture in a subgroup of children whose stool Xpert results we had previously reported on. However, stool culture was discontinued early due to the high contamination rate relative to its poor diagnostic yield. Stool culture did not confirm any of the children with drug-resistant TB and was positive only in 4% of children overall, mostly children with severe manifestations of PTB.

We found that adding Xpert testing of the same stool specimens increased *M. tuberculosis* detection by 100%, since seven stool specimens were Xpert positive but culture negative or contaminated, thereby adding seven additional confirmed diagnoses to the seven confirmed by stool culture. Although Xpert testing had better sensitivity than culture for stool, Xpert only allows partial DST: combining the two testing methods could improve the sensitivity of stool testing, while simultaneously enabling at least a portion of specimens to undergo full DST if clinically relevant. In our cohort, none of the children with MDR-TB were detected using either stool culture or stool Xpert. This may be due to chance since the numbers were small. Studies enrolling children with suspected drug-resistant TB would be best placed to opportunistically evaluate the diagnostic utility of stool culture and Xpert in this patient population.

The sensitivity of stool culture compared to mycobacteriological confirmation using respiratory specimens was 24%, excluding contaminated cultures. If these are included as "not positive," the sensitivity was even lower at 16.2%. This is because some of children excluded based on contaminated stool culture in the first calculation had confirmed TB, so the relative proportion of stool culture-positive children was higher (data not shown). Allowing stool to be collected after TB treatment initiation (up to 7 days) is a limitation of this study and may have contributed to lower bacillary numbers in stool. In contrast, according to protocol entry criteria, respiratory specimens were

mostly collected pretreatment. Although our data suggest that pretreatment collection of stool was not associated with a higher proportion of positive stool cultures, this analysis was not adequately powered. Notably, all culture-positive stools came from children whose stool was collected no more than 1 day after the first culture-positive respiratory specimen. This is probably a function of a correlation between stool and sputum mycobacterial loads. Collection of stool a few days after respiratory specimens may result in lower detection from stool due to a variety of factors, such as treatment with antibiotics and antituberculosis drugs and reduced sputum production leading to less sputum being swallowed.

An additional limitation of our study, which may have negatively biased the stool culture results, is that a single stool specimen was compared to multiple respiratory specimens. We could not find any published studies evaluating the incremental diagnostic yield of additional stool specimens, but it is plausible that, similarly to respiratory specimens, increasing the number of specimens collected could have an additive effect. Although mindful of these limitations, we opted for a pragmatic approach to stool collection, since we were evaluating the potential of stool testing as a feasible strategy for resource-limited settings. Applying excessively strict conditions for method and timing of collection would severely limit feasibility and is less likely to be applicable on the field. Although the cost of a second culture may be prohibitive in many settings, the value of a second stool culture should be studied and considered for settings where this may be an option.

The sensitivity of stool culture in our study, although low, is higher than that reported in the other pediatric studies on stool culture for TB diagnosis by Donald and Oberhelman, where the sensitivity was 15 to 20% compared to culture of two gastric aspirates (20, 21, 30). Our study applied relatively narrow entry criteria, which resulted in almost 50% of the cohort initiating TB treatment, and in a considerable proportion having bacteriologically confirmed TB (42%). In absolute numbers, our study had almost double the number of bacteriologically confirmed cases compared to the other three studies, likely indicating a more severe spectrum of TB disease in our hospital-based cohort. The high prevalence of disease resulted in a high positive predictive value of stool culture (85%), which may not be generalizable to all settings and will be highly dependent on the selection criteria for investigation and on the expected prevalence and severity of TB disease.

It is also difficult to compare our results to the other pediatric studies, as different protocols for stool preparation and different culture methods were used. Oberhelman et al. used a small initial stool mass (0.1 g) diluted in 6 ml of phosphate-buffered saline (PBS) (20, 30), whereas Donald et al. combined two stool specimens (final mass not specified) (21) and followed the method published by Allen (31). All three studies used 1% final concentration sodium hydroxide (NaOH) for decontamination, followed by centrifugation. For culture, Donald et al. used the Bactec radiometric culture, while Oberhelman et al. used both Lowenstein-Jensen and microscopic observation drug susceptibility methods. We used a Mycobacteria Growth Indicator Tube (MGIT; Becton Dickinson, Sparks, MD) culture with PANTA, which is more sensitive than solid culture (32) and is the method used by the South African National Health Laboratory Service. However, despite the addition of antibiotics, MGIT culture is more prone to contamination by commensal microorganisms (32). The abundant microflora which constitutes stool grows rapidly in culture and prevents the identification of the slower-growing *M. tuberculosis* bacilli. The other published pediatric stool culture studies do not report on contamination rates, but studies in adults using MGIT report 14 to 38% contaminated cultures (23, 33, 34). Earlier reports using nonselective culture media on stool samples resulted in excessive contamination, for the detection of both *M. tuberculosis* (35) and *Mycobacterium avium* complex (MAC) (36, 37), leading to early discontinuation of those protocols. More recently, liquid culture has become widely available and is known to result in higher contamination for sputum and nonsputum samples than solid culture media (32).

Culture contamination was the main reason for discontinuing stool culture in our

study. Despite instructing caregivers to keep stool specimens refrigerated and allowing for a maximum 72 h from collection to processing, we did not collect data on the site of collection (home versus hospital): it is possible that ideal conditions were not maintained for stool specimens collected at home, and that this contributed to high contamination rates. Various techniques to reduce stool culture contamination in the laboratory have been evaluated. Allen tested different decontaminating agents and concluded that NaOH was superior to sulfuric acid and alkali precipitation for recovery of *M. tuberculosis* and decontamination (31). In a separate similar study, NaOH also resulted in higher yield and comparable contamination rates compared to Portaels solution and benzalkonium chloride-1-hexadecylpyridinium chloride (35). El Khechine et al. replaced NaOH decontamination with 0.25% chlorhexidine in their laboratory handling of stool samples (19, 38), citing unpublished data of improved recovery versus contamination compared to NaOH (19). Chlorhexidine is inactive against mycobacteria and may increase the recovery of *M. tuberculosis* (39, 40). The sensitivity of stool culture in the study by El Khechine is the highest reported for the diagnosis of PTB at 54% (19).

Allen also tried to reduce stool culture contamination by diluting samples after the 1% NaOH digestion/decontamination procedure, before inoculation into culture medium (31). Dilutions of 1:10 substantially reduced contamination without affecting *M. tuberculosis* yield.

Other stool decontamination methods have been evaluated for the recovery of MAC, including the use of oxalic acid (which resulted in similar contamination rates but improved MAC detection) (36) and testing different concentrations of and time exposure to NaOH (37). Although certain protocols could achieve improved detection, the effect on contamination rates was more variable. Importantly, the pathophysiology of MAC disease in AIDS patients, where disease may be primarily abdominal and rapidly disseminates, may explain the higher sensitivity of stool culture for MAC compared to *M. tuberculosis* in patients with suspected PTB.

Pediatric stool culture studies for *M. tuberculosis* detection have not evaluated higher NaOH concentrations and longer exposure times for sample decontamination, nor the effect of sample dilution. However, it is plausible that these modifications may disproportionately affect mycobacterial recovery compared to reduction of bacterial and fungal overgrowth on the already paucibacillary specimens typically collected from children with PTB.

**Conclusions.** Although stool can easily be collected by caregivers and untrained health care workers, stool sample preparation and processing for culture are relatively complex and laboratory protocols have yet to be optimized. Given the available evidence, stool culture for TB diagnosis cannot currently be recommended to replace culture and Xpert of respiratory samples for the diagnosis of intrathoracic TB in children. Culture remains an expensive technique, and the high percentage of non-evaluable results from contamination using standard protocols paired with limited diagnostic sensitivity does not currently justify its routine use.

More work is needed before stool culture can be promoted as a feasible diagnostic strategy for resource-limited settings. Given the limited options for confirming TB in children from high-burden settings, stool culture may still have a role in TB diagnosis as an adjunctive diagnostic measure or in clinical situations where confirmation of TB disease and DST results is critical, but laboratory research should be prioritized over clinical evaluations. Specifically, promising laboratory protocols that have shown better sensitivity and low contamination rates, such as those using chlorhexidine, should be systematically evaluated and compared to current protocols. Optimized laboratory protocols could then be applied to targeted high-risk pediatric populations such as children at risk of MDR-TB, those with HIV infection and those with severe forms of intrathoracic TB, where diagnosis is most critical. This approach would ultimately inform whether stool culture has a place in resource-limited settings with laboratory capacity but inadequate resources for sputum collection in children.

## MATERIALS AND METHODS

Cohort eligibility, enrollment, and investigation have been previously described (25). In brief, following caregiver written consent, children <13 years of age who presented to two referral hospitals in Cape Town, South Africa, with suspected intrathoracic TB (PTB inclusive of pleural effusion and hilar adenopathy) were consecutively enrolled from April 2012 to June 2014. Eligibility was based on  $\geq 1$  of the following symptoms: (i) cough lasting  $\geq 2$  weeks, (ii) unexplained fever for  $\geq 1$  week, or (iii) poor growth or weight loss over the preceding 3 months. We also included children with any cough duration, if  $\geq 1$  of the following characteristics were present: (i) exposure to an identified TB source case in the preceding 12 months, (ii) positive TST if previously negative or unknown, or (iii) a CXR suggestive of TB as assessed by the study clinician. Infants <3 months of age with pneumonia unresponsive to appropriate antimicrobials or with unexplained and unresponsive sepsis syndrome were also eligible. We excluded children who had received >1 dose of antituberculosis therapy (excluding isoniazid preventive therapy) before respiratory specimen collection, who had an established alternative diagnosis at screening, or who were unable to commit to follow up. Children with both extrapulmonary and intrathoracic manifestations of TB were eligible.

Investigations included TST (Mantoux, two tuberculin units of PPD RT-23; Statens Serum Institute, Copenhagen, Denmark), HIV testing, and CXR, evaluated by two independent experts. TST results were read 48 to 72 h after placement and were considered positive if the wheal measured  $\geq 10$  mm if HIV negative and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccinated,  $\geq 5$  mm if HIV positive, or not BCG vaccinated. Evidence of BCG vaccination was determined by written record in the immunization card or evidence of BCG scar in the right deltoid area. HIV testing was by HIV DNA PCR in children <18 months of age, and by HIV antibody testing (using enzyme-linked immunosorbent assay [ELISA]) if  $\geq 18$  months.

Treatment decisions were made by the attending clinicians based on clinical, epidemiological, and bacteriological findings, including the results of all respiratory and stool specimens collected by the study team. For research purposes, participants were classified using the revised clinical case definitions of intrathoracic TB in children (27), into the categories "confirmed TB" (*M. tuberculosis* confirmed by culture or Xpert on at least one respiratory specimen or on stool Xpert), "unconfirmed TB" (no bacteriological confirmation of *M. tuberculosis* and a minimum two of well-defined TB symptoms; proof of TB infection; TB exposure in the past 12 months; CXR suggestive of TB; and favorable response to anti-tuberculosis treatment at 2 months), and "unlikely TB" (no bacteriological confirmation of *M. tuberculosis* and the criteria for "unconfirmed TB" not met). The classification was determined retrospectively at the 2-month follow-up, when culture results from enrollment were available and treatment response was assessed. Disease severity was determined using a pragmatic modification of a published classification (28), wherein radiologically severe TB cases demonstrated any of the following: (i) complications from typical radiological manifestations of TB (e.g., cavities, expansile pneumonia, and nodal airway obstruction), (ii) bilateral parenchymal involvement, (iii) overall parenchymal involvement more extensive than the total area of the right upper lobe, or (iv) disseminated (miliary) TB. For CXR not typical of TB, criteria 2 and 3 were used to define severe disease.

**Specimen collection, transport, and laboratory methods.** At enrollment, on each of two consecutive days, we collected one specimen of two different types (four specimens in all). Study protocol required two early morning GA samples from young children (<5 years of age) unable to expectorate or two fasting SPT in older children, as well as two IS samples with or without nasopharyngeal suctioning. Standard procedures were followed (25). Some children had additional respiratory specimens collected as clinically relevant. One stool specimen per child was collected within 7 days of enrollment. Caregivers were given verbal and written instructions on how to collect stool. For young children in diapers, a urine bag placed onto the perineum prevented urine from mixing with stool. Formed stool was collected directly from the diaper using a scoop attached to the lid of the stool collection receptacle. For liquid stool, the diaper was fitted inside out or plastic waterproof briefs were fitted under the child's diaper in direct contact with the skin: as soon as stool was passed, the liquid stool was poured or scooped into the stool receptacle. Children who were toilet trained were each given a clean potty into which to pass stool, or they could pass stool onto a sheet of plastic cling film fitted onto a conventional toilet seat. Stool receptacles were premarked to indicate the amount of stool needed (six scoops, equivalent to 2 to 3 g).

Specimens collected in hospital were kept refrigerated and transported to the laboratory in a cool box within 4 h of collection. For stool collected at home after discharge from hospital, caregivers were instructed to keep the specimens in a fridge. The study team was notified by phone when the stool sample was ready, and each specimen was collected from the participant's home. Stool specimens were stored refrigerated for maximum 72 h from collection to processing.

Respiratory specimens were processed at the National Health Laboratory Service Microbiology Laboratory at Tygerberg Hospital. For digestion/decontamination, *N*-acetyl-L-cysteine (NALC)-NaOH was used (final NaOH concentration, 1.25%), before concentrated fluorescent Auramine-O smear microscopy (41), Xpert MTB/RIF, and liquid MGIT culture.

Stool specimens were homogenized with 20 ml of PBS (pH 6.8) by vortexing: 5 ml of the stool-PBS mixture was then processed with NALC-NaOH (1.25%). Similarly to respiratory specimens, after concentration at  $3,000 \times g$  for 20 min, the pellet was resuspended in 2 ml of PBS: a drop was used for fluorescent Auramine-O smear microscopy (41), 0.5 ml was used for MGIT culture (32), and 1.0 ml was mixed with 2.0 ml of Xpert sample reagent and loaded into the GeneXpert instrument (software v4.4a) according to manufacturer's instructions.

Smears were graded according to the WHO/International Union Against Tuberculosis and Lung Disease system (42). Cultures were incubated for up to 42 days. If no growth was observed, cultures were declared negative. For positive cultures, the time to positivity in days was noted, and a

Ziehl-Neelsen (ZN) stain was performed on the culture. If the culture was ZN positive, mycobacterial identification and drug susceptibility for isoniazid and rifampin were completed using MTBDRPlus (Hain Life Science, Nehren, Germany). Rifampin-resistant strains underwent phenotypic DST for ofloxacin and amikacin using the agar proportion method. If growth of bacteria/fungi was observed on blood agar plates and/or non-acid-fast bacteria were seen on the ZN smear, the MGIT culture was considered contaminated. Contaminated cultures from respiratory specimens were recontaminated and reincubated once. Contaminated stool cultures were not further decontaminated as local laboratory experience was that recontamination was rarely successful. Contaminating organisms were not identified. The laboratory technician who handled the stool cultures was not blind to other microbiology results.

**Statistical analysis.** The primary objective was to evaluate the sensitivity, specificity, and predictive values of the stool culture for the diagnosis of intrathoracic TB in children, compared to (i) confirmed TB, as defined above, and (ii) a clinical decision to treat. In secondary analyses, we compared the diagnostic utility of stool culture to the culture of (i) two GA or two SPT specimens, the reference standard used in similar published studies, and (ii) respiratory specimens collected on the same day as stool. We also evaluated the incremental diagnostic value of Xpert testing of the same stool specimen, and the combined sensitivity of stool Xpert and culture versus confirmed TB from respiratory specimens.

All analyses of diagnostic accuracy were conducted per patient (not per specimen). Children were included in analysis if they had a minimum of one stool and one respiratory specimen collected and if stool was collected within 7 days of the respiratory specimens. Contaminated cultures and invalid/error Xpert results were considered nonevaluable and were not repeated. For diagnostic accuracy calculations, participants were excluded if stool culture was contaminated or if all the results of the respiratory specimens were nonevaluable.

Clinical and demographic characteristics were summarized by clinical case definitions using means and standard deviations if normally distributed and with medians and interquartile ranges if not normally distributed. The chi-squared test and Fisher exact test were used for comparisons between proportions. STARD guidelines were followed for reporting and analyses (43). Analyses were generated using Stata 14.0 special edition software (Stata statistical software, release 14; StataCorp LP, College Station, TX).

This study was approved by the Stellenbosch University Health Research Ethics Committee (reference N11/09/282) and by local health authorities.

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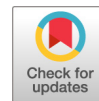
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# Culturing Stools To Detect *Mycobacterium tuberculosis*

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**KEYWORD** tuberculosis

A recent study by Elisabetta Walters and collaborators evaluated stool culture for the diagnosis of pulmonary tuberculosis in children in South Africa (1). It indicated that among 37 children with bacteriologically confirmed pulmonary tuberculosis, only 6 (16.2%) had *Mycobacterium tuberculosis* in their stools, while 1 child that was classified as having “unconfirmed” tuberculosis also had *M. tuberculosis* in his stools and provided the only positive collected specimen. This disappointing result contrasts with our experience comprising mainly adults with microbiologically confirmed *M. tuberculosis* (2, 3). I noted several protocol discrepancies which may account for the disappointing results. With regard to the specimens used for the diagnosis, Elisabetta Walters and collaborators collected liquid stools directly from diapers from an unspecified number of the 37 children. So far, only formed stools have been evaluated for diagnostic value for pulmonary tuberculosis, not liquid stools recovered from diapers (2, 3). Therefore, it is not possible to use liquid stools collected from diapers for the diagnosis of pulmonary tuberculosis in children; this will require a first-step evaluation. The protocol used by Elisabetta Walters and collaborators diluted, on average, 100-mg stool specimens in a 7-ml Bactec bottle instead of plating this material onto any appropriate solid culture medium (4). This protocol may have diluted the inoculum down to the point where *M. tuberculosis* is just not able to grow at all. Indeed, Elisabetta Walters and collaborators used the century-old decontamination protocol proposed by Petroff in 1915 (5), and 12 of the 37 (32.4%) stool specimens yielded contaminated culture. I demonstrated that the chlorhexidine-based decontamination protocol is superior to other protocols with regard to decontamination of stools (4). However, using chlorhexidine-based decontamination implies the use of a culture medium containing egg lecithin, because chlorhexidine can be used only with solid medium, such as egg-containing medium (Löwenstein-Jensen or Coletsos medium). The one series that my colleagues and I developed specifically contains egg lecithin (6). All together, the 12 contaminated cultures with the 7 positive cultures led to culture positivity for 19 out of the 37 (51.3%) children, including babies; diarrheal stools for which no culture protocol for tuberculosis had ever been evaluated were omitted.

I agree with the authors’ conclusion that culturing stools for *M. tuberculosis* requires adequate protocols. I do not agree with the assertion that the very basic sample culture protocols are more expensive than some commercially available molecular tests. I also plead for the replacement of the disputable gastric aspirate testing by the collection of stools, which I believe complement or replace respiratory tract samples for the diagnosis of pulmonary tuberculosis in adults and children (7).

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For the author reply, see <https://doi.org/10.1128/JCM.00056-18>.



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## Reply to Drancourt, "Culturing Stools To Detect *Mycobacterium tuberculosis*"

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We recently evaluated the diagnostic utility of culture of stool compared to culture of respiratory specimens in young South African children suspected of having pulmonary tuberculosis (TB) (1). We reported a high proportion of culture contamination (41.5%) and low sensitivity (33.3%) for stool compared to results from culture of respiratory samples, mostly gastric aspirates (GA) and induced sputum.

Similarly to Drancourt (2) and others (3, 4), we acknowledge the need for less invasive specimen collection techniques for children, which has been the motivation for our work on the use of stool (1, 5, 6). We agree with Drancourt (7) that his chlorhexidine-agar plate culture medium protocol on stools shows promise compared to *N*-acetyl-L-cysteine (NALC)-NaOH with MGIT culture, with improved recovery of *Mycobacterium tuberculosis* (19% versus 9%) and reduced contamination rates (9% versus 17%) (8). We also agree that the use of different culture media (including the recently published MOD9 medium) may improve the yield from stool cultures and that more research is warranted, especially in children.

However, there is insufficient evidence in the published literature indicating that stool culture is a better diagnostic tool than GA for TB diagnosis in children.

The first paper cited by Drancourt (9), a retrospective study, included only 39 patients from whom stool was collected, 15 of whom had GA samples taken and 8 of whom had both sample types taken, precluding a head-to-head comparison of these two sampling strategies. Of the 9 patients (all but 1 were adults) with positive GA cultures, 4 had stool collected, of which 3 were positive (75%). Stool was collected from 4 of 6 patients with negative culture of GA, one of whom was stool culture positive. The other study referenced is a case report of a single adult with laryngeal TB, whose stool, but not respiratory specimens, was culture positive for *M. tuberculosis*. No GA was collected (10).

Other studies by Drancourt's group have evaluated various respiratory specimens, but not GA, versus stool culture directly (8, 11, 12). These studies mostly excluded children, and some did not investigate patients in a standard manner (8, 12).

Published diagnostic studies, including our work (13–15), investigating children by standard protocols that allow for direct comparison of culture yield between GA and stool all demonstrate the lower sensitivity of culture from stool than from GA.

We are highly supportive of research which might demonstrate that stool culture using the chlorhexidine protocol is equivalent to or better than culture of respiratory specimens for young children, who are unable to expectorate sputum and for whom a diagnosis of tuberculosis is most challenging.

Our study was the first to report how liquid versus formed stools were handled. *M. tuberculosis* was recovered from both formed and unformed/liquid stools. It is imprudent to advocate for stool to replace GA if liquid stools have not been evaluated by the

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proposed chlorhexidine protocol, given that most young children will not pass formed stools.

Finally, both molecular tests and culture methods remain expensive in resource-limited settings. Sufficient evidence is needed to justify the cost of tests relative to the diagnostic yield. Until more-adequate data become available, we should not advocate for stools to replace respiratory specimens in children.

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## Chapter 9

# Specimen pooling as a diagnostic strategy for microbiologic confirmation in children with pulmonary (intrathoracic) tuberculosis

### 9.1 Rationale

Detection of *M.tb* in children with suspected PTB is improved by increasing the number<sup>123-125</sup> and variety<sup>108,235</sup> of respiratory specimens collected. Furthermore, collecting specimens over consecutive days may result in a higher cumulative yield than same-day specimen collection<sup>108,109</sup>, but it is not feasible in many clinical settings due to the high cost and the frequent need for hospitalisation. Overnight sputum pooling in adults allows for the collection of higher volume sputum specimens, and has been shown to increase the detection of *M.tb* by smear microscopy and culture<sup>239-242</sup>. This strategy is used mainly in the context of early bactericidal activity trials of novel antituberculosis drugs and regimens in adults with PTB<sup>240</sup>. It is not recommended in routine clinical care due to the higher risk of culture contamination<sup>237</sup>. In young children, pooling together multiple specimens after collection by health care workers has been suggested as an approach to increase the specimen volume and improve microbiological yield<sup>60</sup>, but no studies had evaluated this strategy to date in children.

## 9.2 Study Aims

This study aimed to compare the diagnostic utility (detection of *M.tb* as well as proportion of culture contamination) of multiple respiratory specimens of different types collected on one day and pooled, in the laboratory, before testing by Xpert and liquid culture, vs. testing individual respiratory specimens for each individual child enrolled in the study.

## 9.3 Methods

The study was implemented from May 2014 - March 2017. Children with suspected PTB enrolled in the parent study (See Chapters 3-5), who were unable to expectorate sputum, were included in this pooling sub-study. Specimen collection included a GA, IS, and NPA on each of two consecutive week days. GA, IS, and NPA specimens collected on the second day were pooled for each child individually in the laboratory, after digestion/decontamination. Specimens of different types collected on the same day were pooled, rather than specimens of the same type collected over consecutive days, in order to improve the feasibility of specimen collection in young children. The reconstituted pellets from specimens collected on the second day were combined (pooled) into one centrifuge tube, and vortex-mixed. Individual (day 1) and pooled (day 2) specimens for each child were tested by smear microscopy, Xpert and liquid culture. McNemar's test was used to compare the paired binary diagnostic outcomes of the pooled specimens by culture or Xpert (day 2) and individual specimens (GA/ IS/ NPA culture or Xpert) (day 1), for each child.

## 9.4 Results

Three hundred and four children were analysed; the median age was 15.1 (IQR 9.6-27.2) months, with only 4/304 (1.3%) children >5 years of age. Overall, 44 (14.5%) had *M.tb* confirmed on any of the study specimens. The diagnostic yield for pooled specimens was significantly higher than single IS and single NPA, by culture alone, Xpert alone and by culture and Xpert combined (i.e. any test positive), but it was similar for pooled specimens vs. single GA.

There was no difference in culture contamination between pooled vs. individual specimens of any type. The overall diagnostic yield of pooled specimens vs. all individual specimens combined, was not significantly different, by Xpert, culture, or Xpert/culture combined. Xpert/culture combined detected 35/44 (79.5%) confirmed cases using pooled specimens, vs. 38/44 (86.4%) for all individual day 1 specimens combined. The yield from a single GA was 29/44=65.9%. GA detected 7/44 (15.9%) cases not detected by any other specimen; for pooled specimens, this proportion was 6/44 (13.6%) and for IS, 2/44 (4.5%). NPA specimens did not add any diagnoses that were not already confirmed on other specimen types.

## 9.5 Conclusions

Although pooled respiratory specimens in this study were not superior to GA for the detection of *M.tb* by culture or Xpert, given the good performance of GA, pooling two GA could be considered in future studies. “Front-loading” specimen collection (GA, IS, NPA) on one day was feasible and effective for the detection of *M.tb*,

while a single GA remains a useful specimen for TB testing in young children and as an individual specimen, has a high diagnostic yield.

## **9.6 Citation**

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## **9.7 Candidate's Contribution**

I was responsible for the design, implementation and data collection for this study. I designed the statistical analyses, interpreted the results, drafted and finalised the manuscript after feedback from the other co-authors.

## **9.8 Published Manuscript**



## **Specimen pooling as a diagnostic strategy for microbiologic confirmation in children with intrathoracic tuberculosis**

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Key words: pulmonary tuberculosis; pediatric; diagnosis; specimen pooling

Abbreviated title: Sample pooling to diagnose pediatric pulmonary TB

Running title: Diagnosis of pediatric pulmonary TB

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## **Abstract**

304 young children with suspected pulmonary tuberculosis had a gastric aspirate, induced sputum and nasopharyngeal aspirate collected on each of two consecutive weekdays. Specimens collected on the second day were pooled in the laboratory for each child individually. The diagnostic yield by Xpert and culture from pooled specimens was not significantly different from that of a single gastric aspirate.

## **Background**

Intrathoracic (pulmonary) tuberculosis (PTB) in children is largely paucibacillary. In young children who cannot expectorate, specimen collection requires gastric aspiration, sputum induction (with nasopharyngeal suctioning), or nasopharyngeal aspiration. Respiratory specimens typically contain low concentrations of *Mycobacterium tuberculosis* (*M.tb*) bacilli, resulting in low sensitivity of currently available molecular tests and culture (1).

In children, increasing the number and variety (2-4) of specimens improves the overall diagnostic yield (detection of *M.tb*). Specimen collection over consecutive days may have a higher cumulative yield than same-day collection (3, 5). However, it is less practical and more costly if hospitalization is required. In young children, pooling multiple specimens after collection, in order to obtain a higher volume specimen, has been suggested as an approach to improve the bacteriologic yield (6).

In this study of children with suspected PTB, we compared the diagnostic yield and culture contamination of multiple respiratory specimen types pooled for microbiologic testing, with the yield from individual respiratory specimens for an individual child.

## **Materials and Methods**

This analysis was part of a prospective diagnostic cohort study enrolling children with suspected PTB in Cape Town, South Africa. Eligibility criteria and enrolment investigations have been previously described (7). In brief, children <13 years of age presenting to two public referral hospitals, with history and symptoms of suspected PTB, were consecutively enrolled May 2014 to March 2017 (7). We excluded children who had received >1 dose of antituberculosis therapy before the first day of respiratory specimen collection, those with a convincing alternative clinical diagnosis, children

with only extrathoracic TB or those who lived remotely. The pooling strategy was assessed in children who could not expectorate sputum, typically children <5 years of age.

Investigations included HIV testing, tuberculin skin test (TST; Mantoux, 2 Tuberculin Units PPD RT-23, Statens Serum Institute, Copenhagen) and a chest radiograph (CR; antero-posterior and lateral), evaluated by two independent experts. During the study, there was a global stock-out of TST, leading to a number of children not having the test. Interferon-gamma release assays were not used.

The attending clinicians were responsible for treatment decisions, with all study-related results made available to them. International consensus clinical case definitions were used to classify participants as “confirmed TB”, “unconfirmed TB”, and “unlikely TB” (8). Categories were assigned retrospectively at the 2-month follow-up, following assessment of treatment response and review of culture results.

#### *Specimen collection and laboratory methods*

The study protocol required the collection of one specimen of three different types, on each of two days (SDC1: Figure 1). The standard schedule was an early morning gastric aspirate (GA), a nasopharyngeal aspirate (NPA) and induced sputum (IS). The same order of collection was followed on both days, although specimens for pooling were collected with no time lag between them. On day 1 a minimum of two hours between GA, NPA and IS were observed (SDC2: Document 1\_Specimen collection). In November 2015, NPA collection was stopped, due to low yield of *M.tb* for NPA (SDC3: Table 1) which did not justify the cost of individual testing. NPA was however still collected for the pooled specimen.

Specimens were processed at the National Health Laboratory Service, Tygerberg Hospital following standard protocols. The reconstituted pellets from specimens collected on the second day were combined (pooled) into one centrifuge tube, and vortex-mixed (SDC1: Figure 1). The individual concentrated day 1 specimens and the pooled respiratory specimen were subjected to fluorescent Auramine-O smear microscopy, Xpert MTB/RIF (Xpert: Cepheid, Sunnyvale, CA) and liquid Mycobacteria Growth Indicator Tube (MGIT, Becton Dickinson, Sparks, MD, USA) culture.

GenoType® MTBDR*plus* line probe assay (LPA: Hain Lifescience, Nehren, Germany) was performed on positive cultures for mycobacterial identification.

### *Statistical Analysis*

The paired binary diagnostic outcomes of the pooled specimens by culture or Xpert (day 2) for each child and individual specimens (GA/ IS/ NPA culture or Xpert) (day 1) for each child were compared for marginal homogeneity using McNemar's test. The Benjamini-Hochberg method was used to correct for multiple testing ( $m=34$ ) of the pooled specimens using a false discovery rate of 10%, which corresponded to a  $p \leq 0.04$ . Diagnostic yield was defined as number of children (not specimens) positive for *M.tb* by Xpert or culture. We compared: a) the diagnostic yield of pooled vs. each individual day 1 specimen type separately (pooled vs. GA, pooled vs. NPA, pooled vs. IS); b) the proportion of contaminated cultures and invalid/error Xpert results for pooled vs. individual specimens separately); c) the total diagnostic yield by Xpert and culture (either positive) of pooled specimens vs. the combined yield from day 1 individual specimens (yield by Xpert or culture from any individual specimen). For a) and b), the pooled specimen had to contain the specimen type to which it was compared (per-protocol approach). For c), both a per-protocol (the number and type of specimens in

the pooled specimen being the same as the number and type of day 1 individual specimens) and a pragmatic approach (including all participants) were used.

Stellenbosch University Health Research Ethics Committee (N11/09/282) and local health authorities approved the study.

## Results

In 304 enrolled children (SDC 4: Figure 2), the median age was 15.1 (IQR 9.6-27.2) months (SDC 5: Table 2). Fifty-one of 304 (16.8%) children had confirmed TB: 44 were confirmed by study specimens and 7 on other specimens. Therefore, the total diagnostic yield for the pooling study was 44 children with confirmed TB. Antituberculosis treatment was initiated in 51 (100%) children with confirmed TB, 77/97 (79.4%) with unconfirmed TB and 6/156 (3.8%) with unlikely TB.

*Pooled specimens vs. individual specimen types* (SDC3 Table 1 for overall specimen results and SDC6 Figure 3 for specimen flow).

When comparing pooled specimens to each individual specimen type (SDC3 Table 1), the proportion difference in diagnostic yield for pooled vs. single IS and single NPA was significantly higher for pooled specimens, by culture alone, Xpert alone and culture and Xpert combined (any test positive), but not for pooled vs. single GA.

The proportion difference in contaminated cultures was not significant for pooled vs. any individual specimen type (Table 1).

*The diagnostic yield of pooled specimens vs. the overall yield from individual specimens*

For the per-protocol analysis, 234/304 (77.0%) children who were included had culture on all specimens, while 223 (73.4%) had Xpert on all specimens. For both the per-

protocol and pragmatic analyses, the proportion difference in diagnostic yield, culture contamination and Xpert error rate of pooled specimens vs. all individual specimens combined was not significant (Table 1).

Of the 134 children treated for TB, 16 (11.9%) started treatment before (median 1.5 days) collection of specimens for pooling. Only one had a pooled specimen with negative culture and an individual positive GA culture, while two cases had culture-positive pooled specimens with negative individual specimens.

## **Discussion**

We found that the overall diagnostic yield from pooled specimens (pooled GA, NPA, and IS) was not different from that of a single GA specimen, but was significantly higher than the yield from single IS or single NPA specimen. Pooled specimens and individual GA also had similar incremental yield and proportions of culture contamination, suggesting that the GA contained within the pooled specimen was probably the main contributor to both the diagnostic yield and the contamination rate of pooled specimens.

Pooling may result in high specimen volume, and also allows for sampling of the respiratory tract at different time points. Early studies demonstrated that shedding of *M.tb* bacilli into respiratory secretions may be intermittent (9) and pooling may increase the chance of collecting respiratory secretions which contain higher concentrations of bacilli. GA may be viewed as a naturally “pooled” specimen, as it consists of multiple expectoration and swallowing cycles, collected within the stomach. In contrast, both IS and NPA reflect a single instance of sampling the respiratory tract.

We elected to pool (in the laboratory) specimens of different types collected consecutively in a standard manner, to avoid prolonged periods of fasting, while

allowing for collection on a single day. A same-day collection strategy avoids longer hospital stay, and potential loss to follow-up associated with consecutive-day collection in ambulatory settings.

Although we may have compromised some of the diagnostic potential of pooled specimens by collecting specimens consecutively for pooling with no time interval between specimens, our focus was to reduce cost of laboratory testing and improve feasibility, and long waiting times would not be practicable in routine clinical settings. A small proportion of children started antituberculosis therapy before collection of pooled specimens. However, our data suggest that this did not impact negatively on *M.tb* detection.

As expected in pediatric TB, collecting multiple specimens from children optimizes *M.tb* detection (SDC7: Figure 4). The yield from multiple specimens on a single day was substantial (38/44=86.4%; Table 1). Previous studies have also shown that “front-loading” specimen collection on one day was comparable to consecutive-day collection (3, 10). However, pooling specimens did not have the expected increase in diagnostic yield. Given the good performance of GA, pooling two GA could be considered in future studies.



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**Table 1. Paired comparisons of culture, Xpert and smear microscopy results for Pooled vs gastric aspirate (GA), Pooled vs induced sputum (IS) and Pooled vs nasopharyngeal aspirate (NPA)<sup>1</sup>, and paired comparisons of culture, Xpert and smear microscopy results for Pooled vs the combined yield of GA, IS and NPA by per-protocol and pragmatic approaches**

		Day 1 GA Culture (n=294)		Day 1 IS Culture (n=298)		Day 1 NPA Culture (n=242)		Day 1 Combined cultures, per-protocol (n=234 <sup>2</sup> )		Day 1 Combined cultures, pragmatic (n=304)	
Type of test	Outcome	<i>M.tb+</i>	<i>M.tb</i> <sup>-3</sup>	<i>M.tb+</i>	<i>M.tb-</i>	<i>M.tb+</i>	<i>M.tb-</i>	<i>M.tb+</i>	<i>M.tb-</i>	<i>M.tb+</i>	<i>M.tb-</i>
Pooled Culture	<i>M.tb +</i>	20	8	18	13	9	15	17	5	25	6
	<i>M.tb -</i>	8	258	2	265	0	218	7	205	10	263
	Difference (95% CI)	0.0 (-0.03, 0.03)		0.04 (0.01, 0.07)		0.06 (0.03, 0.10)		-0.01 (-0.04, 0.02)		-0.01 (-0.04, 0.02)	
	McNemar's p-value	1,000		0,005 <sup>3</sup>		<0.001 <sup>4</sup>		0,564		0,317	
		Day 1 GA Xpert (n=281)		Day 1 IS Xpert (n=296)		Day 1 NPA Xpert (n=242)		Day 1 Combined Xpert, per-protocol (n=223 <sup>2</sup> )		Day 1 Combined Xpert, pragmatic (n=304)	
		<i>M.tb+</i>	<i>M.tb-</i>	<i>M.tb+</i>	<i>M.tb-</i>	<i>M.tb+</i>	<i>M.tb-</i>	<i>M.tb+</i>	<i>M.tb-</i>	<i>M.tb+</i>	<i>M.tb-</i>
Pooled Xpert	<i>M.tb+</i>	12	8	14	9	8	11	13	3	19	4
	<i>M.tb-</i>	4	257	1	272	1	222	5	202	5	276
	Difference (95% CI)	0.01 (-0.01, 0.04)		0.03 (0.00, 0.05)		0.04 (0.01, 0.07)		0.00 (-0.04, 0.02)		0.00 (-0.03, 0.02)	
	McNemar's p-value	0,388		0,011 <sup>4</sup>		0,004 <sup>4</sup>		0,478		0,739	
		Day 1 GA Culture/Xpert combined (n=294)		Day 1 IS Culture/Xpert combined (n=298)		Day 1 NPA Culture/Xpert combined (n=242)		Day 1 Combined culture/Xpert,per-protocol (n=234 <sup>2</sup> )		Day 1 Combined culture/Xpert, pragmatic (n=304)	
		<i>M.tb+</i>	<i>M.tb-</i>	<i>M.tb+</i>	<i>M.tb-</i>	<i>M.tb+</i>	<i>M.tb-</i>	<i>M.tb+</i>	<i>M.tb-</i>	<i>M.tb+</i>	<i>M.tb-</i>
Pooled Culture/Xpert combined	<i>M.tb+</i>	22	10	21	14	11	16	20	5	29	6
	<i>M.tb-</i>	7	255	2	261	0	215	6	203	9	260
	Difference (95% CI)	0.01 (-0.02, 0.04)		0.04 (0.01, 0.07)		0.07 (0.03, 0.10)		0.00 (-0.04, 0.03)		-0.01 (-0.04, 0.02)	
	McNemar's p-value	0,467		0,003 <sup>4</sup>		<0.001 <sup>4</sup>		0,763		0,439	
		Day 1 GA Smear (n=294)		Day 1 IS Smear (n=298)		Day 1 NPA Smear (n=242)		NA		NA	
		Positive	Negative	Positive	Negative	Positive	Negative				
Pooled Smear	Positive	3	3	2	4	1	2				
	Negative	1	287	1	291	0	239				
	Difference (95% CI)	0.01 (-0.01, 0.02)		0.01 (-0.01, 0.03)		0.01 (-0.01, 0.02)					
	McNemar's p-value	0,317		0,180		0,157					
		Day 1 GA Culture (n=294)		Day 1 IS Culture (n=298)		Day 1 NPA Culture (n=242)		Day 1 Combined cultures, per-protocol (n=234 <sup>2</sup> )		Day 1 Combined cultures, pragmatic (n=304)	

		Not Contaminated			Contaminated					Not Contaminated			Contaminated			
		Contaminated	Not Contaminated	Contaminated	Contaminated	Not Contaminated	Contaminated	Contaminated	Contaminated	Not Contaminated	Contaminated	Not Contaminated	Contaminated	Not Contaminated	Contaminated	
Pooled Culture	Contaminated	3	18	1	20	2	12	2	13	5	16	2	13	5	16	
	Not Contaminated	16	257	9	268	5	223	23	196	27	256	23	196	27	256	
	Difference (95% CI)	0.01 (-0.04, 0.05)			0.04 (0.00, 0.08)			0.03 (-0.01, 0.07)			-0.04 (-0.10, 0.01)			-0.04 (-0.08, 0.01)		
	McNemar's p-value	0,732			0,041 <sup>4</sup>			0,090			0,096			0,093		
		Day 1 GA Xpert (n=281)			Day 1 IS Xpert (n=296)			Day 1 NPA Xpert (n=242)			Day 1 Combined Xpert, per-protocol (n=223 <sup>2</sup> )			Day 1 Combined Xpert, pragmatic (n=304)		
		Error	Not Error	Error	Not Error	Error	Not Error	Error	Not Error	Error	Not Error	Error	Not Error	Error	Not Error	
Pooled Xpert	Error	0	3	0	3	0	3	0	3	0	3	0	3	0	3	
	Not error	0	278	2	291	0	239	2	218	2	299	2	218	2	299	
	Difference (95% CI)	0.01 (0.00, 0.03)			0.00 (-0.01, 0.02)			0.01 (-0.01, 0.03)			0.00 (-0.02, 0.03)			0.00 (-0.01, 0.02)		
	McNemar's p-value	0,083			0,655			0,083			0,655			0,655		

GA: gastric aspirate; IS: induced sputum; NPA: nasopharyngeal aspirate; M.tb: Mycobacterium tuberculosis; CI: confidence interval.

<sup>1</sup>For an explanation for the different denominators used in these comparisons, see SDC4: Figure 1; <sup>2</sup>For the per-protocol analysis, 234/304 (77.0%) children had the same number and type of specimens collected on day 1 and for pooling. Of these, all 234 (100%) had culture on all specimens tested by culture, while 223 (95.3%) had Xpert on all specimens tested by Xpert.<sup>3</sup>M. tb- includes all non-positive results (for culture: contaminated, NTM and negative; for Xpert: error/invalid or negative); <sup>4</sup>Using the Benjamini-Hochberg method,  $P \leq 0.04$  was defined as statistically significant (24).

## Supplementary digital content legend

SDC1. Figure 1. Specimen pooling strategy for each individual child

SDC2. Word document: Schedule of events and specimen collection

SDC3. Table 1. Diagnostic test results for children with suspected TB for culture, Xpert and smear microscopy tests displayed for 304 participants with gastric aspirate (GA), induced sputum (IS), nasopharyngeal aspirate (NPA) and pooled respiratory specimens

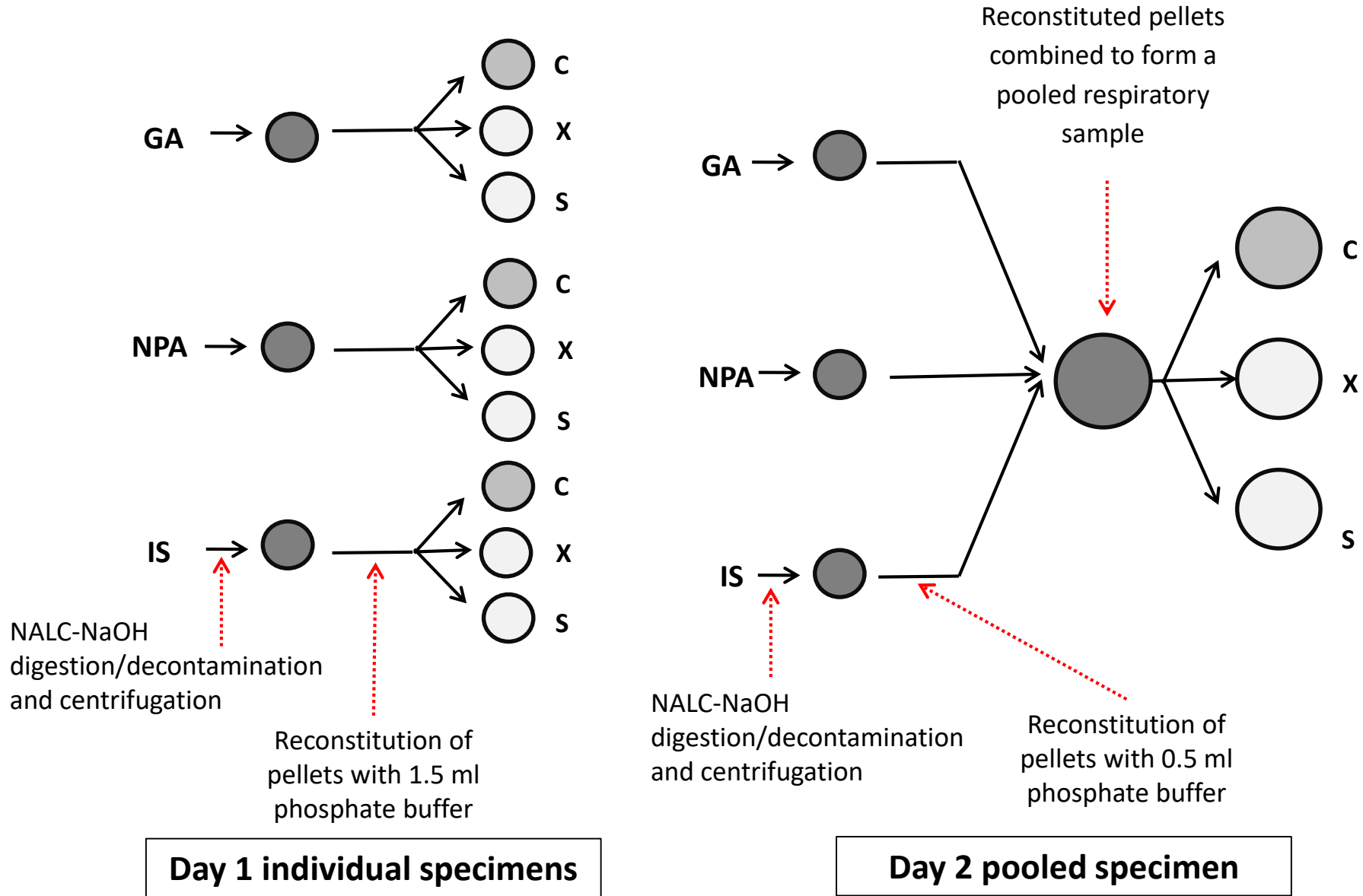
SDC4. Figure 2. Cohort flow diagram

SDC5. Table 2. Cohort characteristics in children with suspected pulmonary tuberculosis

SDC6. Figure 3. Sample flow for the comparison between pooled and individual sample types

SDC7. Figure 4. Incremental yield of different specimen types by culture, Xpert MTB/RIF and culture/Xpert MTB/RIF combined.

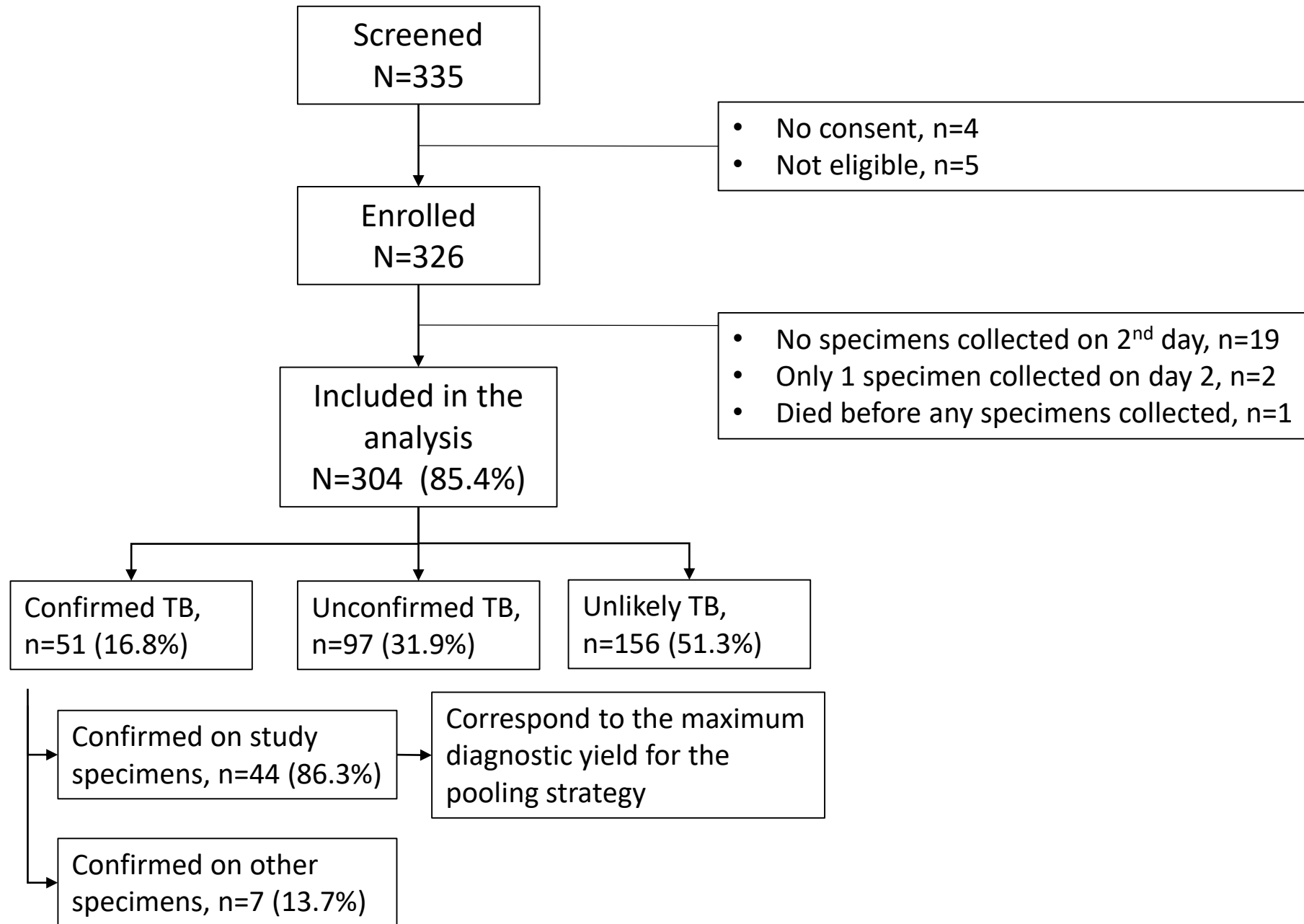
Figure 1. Specimen pooling strategy for each individual child



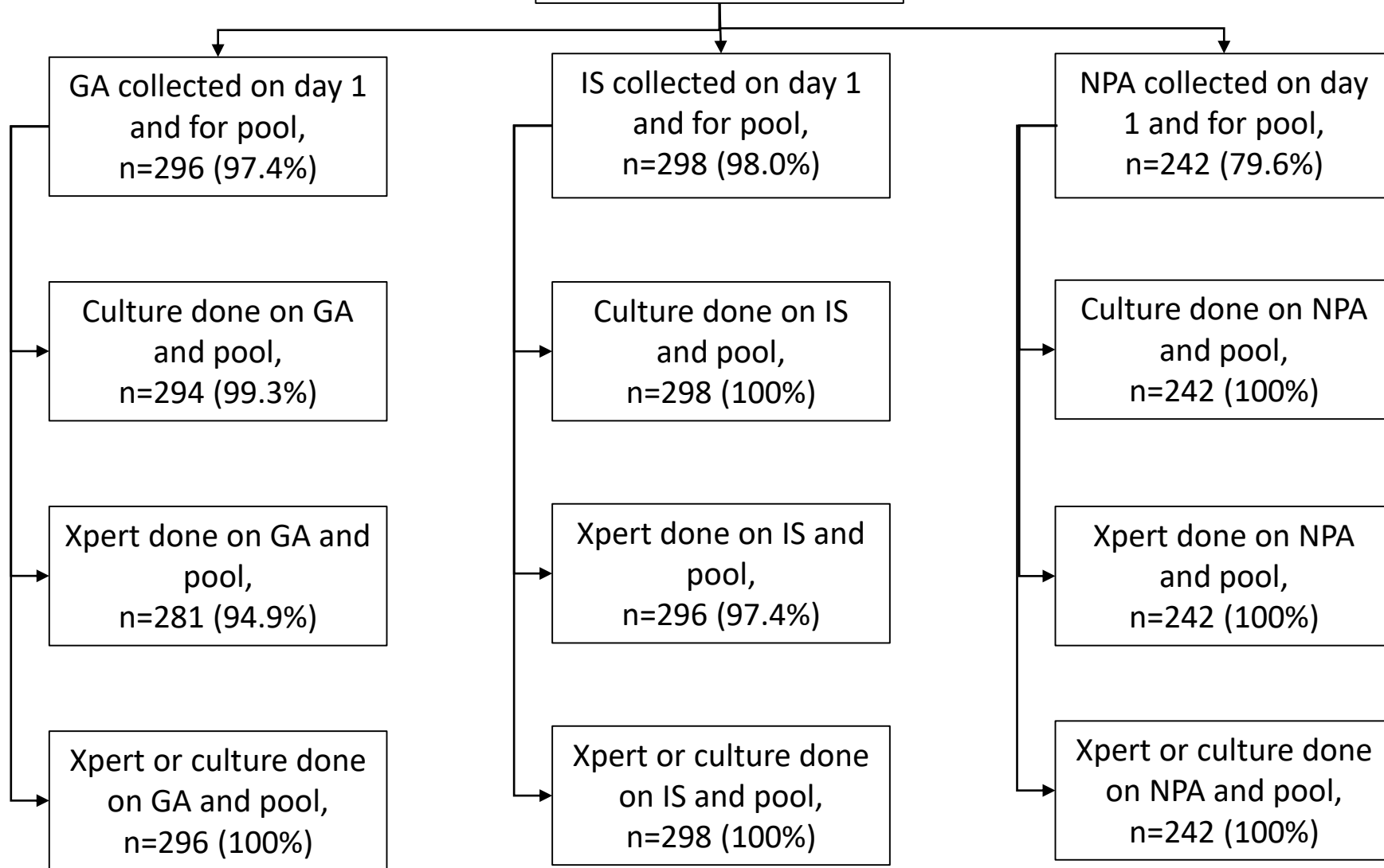
GA: gastric aspirate; NPA: nasopharyngeal aspirate; IS: induced sputum; C: MGIT culture; X: Xpert MTB/RIF; S: smear



Figure 2. Cohort flow diagram



SDC6: Figure3. Sample flow for the comparison between pooled and individual sample types



GA: gastric aspirate; IS: induced sputum; NPA: nasopharyngeal aspirate

Table 1. Pooling Study Schedule of Events

	Enrolment	
	Day1	Day2
History and clinical exam	X	
CXR dual read	X	
TST	X	
Adult source tracing	X	
HIV ELISA <sup>a</sup>	X	
Gastric Aspirate (GA) for Smear, Xpert, MGIT <sup>b</sup>	X	
Nasopharyngeal aspiration (NPA) for Smear, Xpert, MGIT	X	
Induced Sputum (IS) for Smear, Xpert, MGIT	X	
Respiratory samples pooled for Smear, Xpert, MGIT #1) GA #2) NPA #3) Sputum induction with suction		X

<sup>a</sup>HIV ELISA to be done unless a recent HIV test on the laboratory system and no risk factors for subsequent HIV exposure since last test. HIV Rapid Test would be performed only if not enough blood can be collected for the HIV ELISA. If ELISA test is positive in a child <18 months of age, blood must be collected for HIV DNA PCR as soon as possible to confirm HIV infection definitively.

HIV PCR to be done as the initial test if the child: a) is <18 months old; b) has had documented HIV exposure pre- or post-natally and c) does not have a documented record of a previous negative HIV PCR test OR if there is ongoing exposure (e.g. breastfed)

<sup>b</sup>MGIT=Mycobacteria Growth Indicator Tube Culture

### Minimum time fasting before procedures

Table 2 Minimum time fasting before collection of respiratory samples

Day 1		
Order	Sample type	Minimum hours nil per os (NPO)
1	Gastric aspirate (GA)	4 hours (preferably overnight fast)
2	Nasopharyngeal aspirate (NPA)	2 hours
3	Induced sputum	2 hours
Day 2 (for pooling)		
Order	Sample type	Minimum hours NPO
1	GA	4 hours (preferably overnight fast)
2	NPA	As soon as GA collected
3	Induced sputum	As soon as NPA collected

Note that the procedures should be performed in the order listed in the above table

## Specimen collection

### 1. Gastric aspiration

#### Equipment required

1. Gloves, particulate respirator masks (N95 or equivalent), disposable aprons
2. Sputum specimen container (screw-top)- preferably Falcon tube
3. Laboratory forms
4. Laboratory bags
5. Nasogastric tube-NGT (6-10 French): preferred are Ryles (longer) or Levin tubes
6. 5, 10 and 20 cc syringe
7. Litmus paper/ pH strips
8. Sodium Bicarbonate (4%) solution for bedside neutralization
9. 3 bed sheets or surgical drapes: one for the bed, one for wrapping child into and one for putting over the child
10. Dropper
11. Normal saline (0.9% NaCl) or sterile water in single use vials

#### Performing gastric aspiration

This procedure is routinely performed by the study nurse or ward nursing staff.

2. The parent/ legal guardian will be instructed regarding overnight fasting of at least 4 hours before early morning gastric aspirate (GA). The procedure is preferably performed early in the morning when the child comes for a study visit or in the ward if child is an in-patient. The procedure may also be performed during the daytime, as long as the child has been kept nil per os (NPO) for minimum 4 hours.
3. Use an assistant (counselor) to help as this procedure requires 2-3 people.
4. Prepare all equipment for the procedure.
5. Disinfect all working surfaces including bed. Place a drape over the bed. Use one drape to immobilize the child and one to cover the child leaving head exposed.
6. Position the child in decubitus supine position with the help of an assistant.
7. Optional: instill 2 drops of oxymetazoline into each nostril to induce vasoconstriction and prevent epistaxis.
8. Measure the distance of the nasogastric tube to the stomach (from tragus of the ear, to nose, to xiphisternum): this estimates the distance that will be required to insert the tube.
9. Placing child's face in the "sniffing air" position, a nasogastric tube is passed from the nose into the stomach to aspirate gastric contents.
10. Attach a syringe (10 if using Levin or 20 ml if using Ryles tubes) to the nasogastric tube (size 6-10 French, depending on the size of the child).
11. Withdraw (aspirate) gastric contents using the syringe attached to the nasogastric tube.

12. Check NGT position by pushing some air from the syringe into the stomach (3-5ml), and listening with a stethoscope over the stomach.
13. Aspirate stomach contents gently and steadily, with the child in each of 3 positions: head central, left lateral and right lateral. Allow a few seconds before aspirating after changing position. If no fluid is aspirated, push tube 1-2cm deeper or pull out 1-2cm shallower, and aspirate. ANY VOLUME  $\geq 1$ ml is adequate for bacteriological examination. However, attempt to obtain as high volume as possible, aiming for a minimum 5mL, especially from a sick child (where a diagnosis is more important).
14. If  $< 1$ ml is aspirated, a gastric lavage can be performed:
  - a. Sterile water (alternative: preservative free normal saline) 10 ml will be inserted down the tube, left for three minutes, and then aspirated until a minimum of 5-10ml aspirate is obtained.
  - b. If no fluid is aspirated, instill additional 10 milliliters of sterile water and aspirate again. If still unsuccessful, repeat this up to 3 times.
15. Transfer full volume of gastric fluid from syringe into a sterile container (Falcon tube).
16. Titrate volume of 4% sodium bicarbonate using a pipette or syringe and pH strips, adding to the specimen until pH 6-7 is reached (in order to neutralize the acidic gastric contents and so prevent destruction of tubercle bacilli)
17. Clean Falcon tube with alcohol swabs
18. Label sample: sample type and number, date, time, time of neutralization volume of bicarb added, total sample volume.
19. Place in sample bag, seal and put into a cold box for transport to lab.

### **Nasopharyngeal aspiration:**

#### **Equipment required:**

1. Suction apparatus
2. Disposable gloves and P2 respirator masks
3. Paper towels
4. Normal saline (0.9%)
5. Oxymetazoline (optional)
6. Sterile 6/7/8 G mucus extractor or nasogastric catheter
7. Cotton wool
8. Kidney dish
9. Laboratory forms
10. Laboratory bags

#### **Procedure**

This procedure for respiratory sampling in young children will be conducted before sputum induction.

1. The child's nose is cleaned with saline drops and cotton wool. If old enough, the child can be asked to blow the nose into a tissue. If the nasal mucus is too thick to be removed with the

measures above, it can be suctioned prior to nasopharyngeal aspiration. A soft catheter size F6/7 is used for suctioning and is discarded immediately afterwards.

2. One drop of oxymetazoline may be instilled into each nostril to prevent nose bleeds.
3. Two drops of sterile saline are instilled into each nostril
4. The length of the cannula used for aspirating the NPA sample is measured as the distance from nostril to tragus of the ear; then the posterior nasopharynx is suctioned using a soft plastic cannula connected to a mucus trap.
5. Suctioning is activated only when the tip of the cannula is in the posterior nasopharynx. When the cannula is passed through the nostrils (during introduction and extraction), the suction is de-activated.

### **Sputum Induction**

Sputum induction is typically used in patients who are unable to produce sputum spontaneously. The patient inhales nebulised hypertonic saline solution, which liquefies airway secretions, promotes coughing and allows expectoration of respiratory secretions. In young children, nasopharyngeal aspiration is usually required for sputum collection.

#### **Contra-indications/precautions:**

1. As hypertonic saline causes bronchoconstriction, the procedure should only be performed after pre-medication with salbutamol and under medical supervision in patients with asthma or severely impaired lung function.
2. As the procedure induces coughing, it should not be performed in patients in whom severe coughing may be harmful, including patients with:
  - a) Unstable respiratory state: acute respiratory distress, pertussis-syndrome, hypoxia (sats <92% in room air), pneumothorax.
  - b) Unstable cardiovascular status (including untreated cyanotic heart disease)
  - c) Recent surgery: attending surgeon to assess
  - d) Any condition where the patient is unable to protect the airway e.g. depressed level of consciousness

#### **Infection control**

The minimum requirement for sputum induction is a single room with door closed and air exhausted to the outside of the building without recirculation. Ideally, the room should be fitted with an air extractor allowing for generation of negative pressure in the procedure room. A “no-entry” sign should be fitted outside the door for the duration of the procedure.

Staff performing procedure (including child carer if present) must wear the recommended TB respiratory protection (particulate respirators) and disposable gloves when handling sputum specimen.

#### **Equipment required:**

1. Spacer with mask
2. Salbutamol (100µg/puff)
3. Suction apparatus
4. Pulse oximeter
5. Nebuliser with tubing and face mask
6. Hypertonic (3-5%) saline solution
7. Disposable gloves and P2 respirator masks

8. Paper towels
9. Kidney dish
10. Sputum specimen container (screw-top)
11. Laboratory forms
12. Laboratory bags
13. 5ml and 10ml syringes
14. 19G needle
15. Sharps container
16. Sterile 6/7/8 G mucus extractor or nasogastric catheter

**Procedure:**

1. Sputum induction is performed by a research nurse trained in this technique, and is undertaken after a 2–3 h fast.
2. Clinical evaluation form is completed before procedure, documenting general observations and chest auscultation. (See section 7 below). Detection of severe respiratory distress or severe tachycardia is a contra-indication for the procedure.
3. Oxygen saturation and pulse rate must be monitored throughout the procedure. Stop the procedure in event of a fall in saturation <90% and a pulse rate >180 or <100 bpm
4. Child is pretreated with 200µg salbutamol via metered dose inhaler with attached spacer to prevent bronchoconstriction. This is done by placing the assembled metered dose inhaler/spacer/mask onto child's mouth and nose. Child is allowed to settle until breathing freely. One puff is activated, keeping the mask in the same position, and the child is allowed to breathe 4-5 times. Mask is removed.
5. The child's nose is cleaned with saline and cotton wool to remove nasal mucus prior to sputum induction. If nasal secretions are thick, a soft catheter size F6/7 is used for suctioning and is discarded immediately afterwards.
6. A jet nebuliser attached to oxygen at a flow rate of 5-7 L per minute delivers 5 mL of 5% sterile saline for 15 minutes **or until child starts to cough**.
7. **In young children who cannot expectorate spontaneously:**
  - a. Once the child starts to cough, sputum is obtained by suctioning through the nasopharynx with a sterile mucus extractor of catheter size 6 or 7. Pass the catheter up to a length equal to the distance from nostril to tragus of the ear, without applying suction. Once in position, apply suction. Catheter can be moved a little and turned to facilitate cough reflex and aspiration of sputum.  
Suction pressures by ages, according to the American Association for Respiratory Care (AARC) Guidelines for appropriate sub-atmospheric nasotracheal suctioning pressures:
    - Neonates: 60 - 80 mm Hg (0.079- 0.10 Bar)
    - Infants: 80 - 100 mm Hg (0.079- 0.13 Bar)
    - Children: 100 - 120 mm Hg (0.13- 0.15 Bar)
    - Adults: 100 - 150 mm Hg (0.13- 0.19 Bar)

Negative pressures should not exceed 150 mm Hg as higher pressures have been shown to cause trauma, hypoxemia and atelectasis.

Once sputum is obtained, the catheter is withdrawn from the nose. DO NOT aspirate as the catheter passes through the nose. If mucus is to be obtained from the oral cavity as

well, the mouth should be rinsed/wiped with anti-bacterial, non-alcohol-based mouth wash prior to the entire procedure, in order to avoid contamination.

- b. If the child does not cough after nebulisation, chest percussion is done over the anterior and posterior chest wall. Mucus is then extracted as (7) above. Nebulisation and chest percussion followed by nasopharyngeal aspiration can be repeated if an inadequate sample is obtained (volume <1mL).

**8. In older children who can expectorate spontaneously:**

- a. Once the child starts to cough, encourage the expectoration of sputum into a sputum container. The child should continue to expectorate until no more sputum can be produced. Nebulisation can be repeated if an inadequate sample is obtained (volume <1mL or watery sample indicative of saliva)
  - b. If the child does not cough after nebulisation, encourage the child to perform deep breathing. The child can also be made to jump or run on the spot if clinically stable and able to do so. Chest percussion is done over the anterior and posterior chest wall. Encourage the child to expectorate as 8a. above.
9. Specimens will be transported directly to the laboratory for processing.
  10. Spacers and nebuliser equipment will be sterilised after use in every patient.
  11. Complete clinical evaluation form after procedure. Any new signs of respiratory distress not settling after 5-10 minutes of supplemental oxygen via face mask must be reported immediately to attending clinician. SAE form must then be completed.

**1.1. Quality Control (QC) of samples**

- a) All forms must be checked and signed-off as correct before they depart from the clinical site. This is done by an appropriate responsible party which may be a study nurse, counsellor, doctor or the study coordinator.
  - a. If the form has not been filled out to satisfaction, it must be returned to the person who filled it in.
  - b. Once a person has signed as "QC," the responsibility of the form's accuracy and completeness lies with the "QC-er".

**1.2. Transport boxes**

- a) At the beginning of every day, ice bricks must be collected in the freezer in room XXXX and packed into the appropriate transport boxes housed in room YYYY. These must be taken to the procedure room at the hospital at the start of the day on days when sampling is planned.
- b) At the end of each day the ice bricks must be returned to room XXXX.
- c) Transport boxes should be returned to room YYYY and wiped clean on the inside and outside at the end of each day using an alcohol based disinfectant. Leave open to dry. Also clean the ice bricks the same way.
- d) Transport boxes and ice bricks can also be kept in the procedure room, as long as they are clean at the end of each day. The boxes should not be left open when respiratory samples are collected and should be wiped after the procedure as well.



### 1.3. Sample transport

- a) Once the samples have been collected, begin the transport procedure by checking that all required forms have been completed and the barcodes match across forms as well as on the samples being transported. If there are any errors here, return the forms to the person who completed them, for correction.
- b) Place samples into a transport box that has the correct temperature.
- c) Forms:
  - a. Complete the Departure site of the Transport Log and re-check that all samples listed there are in the transport box.
  - b. Depart the site with all the appropriate forms (including Requisition Forms and Transport Log).
  - c. On arrival at the laboratory, complete the Transport Log by filling in the Arrival side of the form. Make sure that there is a person present to receive the sample, this person signs to verify sample reception.
- d) Leave the sample and the requisition form in this person's possession.

Table 3 Volumes, Storage and Transport Conditions for Sample Types

Sample	Minimum Volume	Temperature to keep until arrival in lab	Storage in Cough Room until transport to lab	Transport to lab	Max time between collection and arrival in lab <sup>A</sup>	Destination Lab
GA	Take time to collect 5-10 mL ideally (1mL min)	2 to 8°C	Fridge	with ice brick	Same day	NHLS TB Lab (9 <sup>th</sup> floor) <sup>D</sup>
All other respiratory samples	As much as possible, minimum 1 mL	2 to 8°C	Fridge	with ice brick	Same day	NHLS TB Lab (9 <sup>th</sup> floor)
HIV ELISA Test	Minimum 0.6 ml	18°C to 25°C	NHLS Tube rack	18°C to 25°C	n/a	NHLS Serology
HIV PCR Test	Minimum 0.5 ml (0.25 ml accepted)	18°C to 25°C	NHLS Tube rack	18°C to 25°C	n/a	NHLS Virology

**Supplemental Digital Content 3: Table 1. Diagnostic test results for children with suspected TB for culture, Xpert and smear microscopy tests displayed for 304 participants with gastric aspirate (GA), induced sputum (IS), nasopharyngeal aspirate (NPA) and pooled respiratory specimens**

Type of laboratory test	Result	Sample Type			
		Pooled n=304	GA n=301	IS n=301	NPA n=242
Culture	Positive for <i>M.tb</i> <sup>1</sup> (%)	31 (10.2)	29 (9.6)	20 (6.6)	9 (3.7)
	Positive for NTM (%)	4 (1.3)	2 (0.7)	4 (1.3)	5 (2.1)
	Negative (%)	248 (81.6)	251 (83.4)	267 (88.7)	221 (91.3)
	Contaminated (%)	21 (6.9)	19 (6.3)	10 (3.3)	7 (2.9)
Xpert	Positive for <i>M.tb</i> (%)	23 (7.6)	17 (5.6)	15 (5.0)	9 (3.7)
	Negative (%)	278 (91.4)	271 (90.0)	282 (93.7)	233 (96.3)
	Error <sup>2</sup> (%)	3 (1.0)	0 (0.0)	2 (0.7)	0 (0.0)
	Not done (%)	0 (0.0)	13 (4.3)	2 (0.7)	0 (0.0)
Positive <i>M.tb</i> on culture or Xpert <sup>3</sup>		35 (11.5)	30 (10.0)	23 (7.6)	11 (4.6)
Smear microscopy	Positive (%)	6 (2.0)	4 (1.3)	3 (1.0)	1 (0.4)
	Negative (%)	298 (98.0)	297 (98.7)	298 (99.0)	241 (99.6)

GA: gastric aspirate; IS: induced sputum; NPA: nasopharyngeal aspirate; *M.tb*: *Mycobacterium tuberculosis*; NTM: non-tuberculous mycobacteria; <sup>1</sup>Positive *M.tb* and contaminated are reported as positive for *M.tb* only by the laboratory; <sup>2</sup>No Xpert invalid results were reported; <sup>3</sup>This parameter represents the diagnostic yield as defined by the study

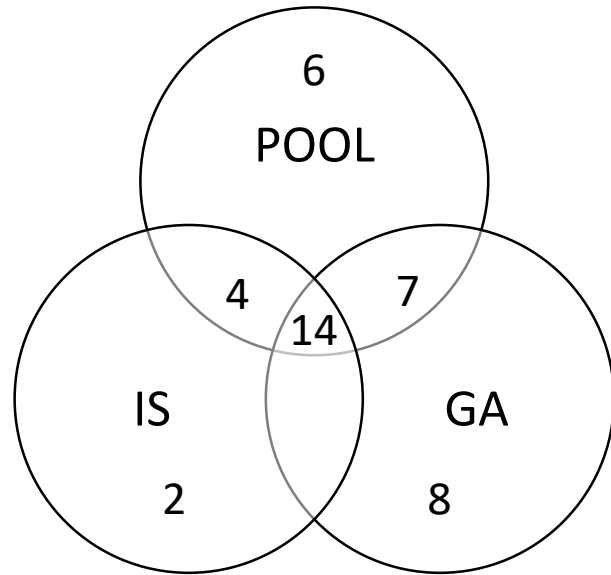
Supplemental digital content 5: Table 2. Cohort characteristics in children with suspected pulmonary tuberculosis

	All participants (n=304)
Median age in months at enrolment (IQR)	15.1 (9.6, 27.2)
Male (%)	169 (55.6)
HIV-positive (%) [n=303 <sup>1</sup> ]	27 (8.9)
Previous antituberculosis treatment (%)	22 (7.2)
Median WAZ (IQR)	-1.39 (-2.56 to -0.48)
WAZ <-2.0 (%)	110 (36.2)
Evidence of BCG vaccination <sup>2</sup> (%)	285 (93.8)
Tuberculin skin test positive (%) [n=225 <sup>3</sup> ]	54 (24.0)
Confirmed TB [n=36]	24 (66.7)
Unconfirmed TB [n=68]	25 (36.8)
Unlikely TB [n=121]	5 (4.1)
Chest radiograph suggestive of TB (%)	79 (26.0)
Severe TB disease (18,20) [n=79]	32 (40.5%)
<b>TB consensus case definition (8):</b>	
Confirmed TB (%)	51 (16.8)
Unconfirmed TB (%)	97 (31.9)
Unlikely TB (%)	156 (51.3)
Treated for TB (clinical decision) (%)	134 (44.1)

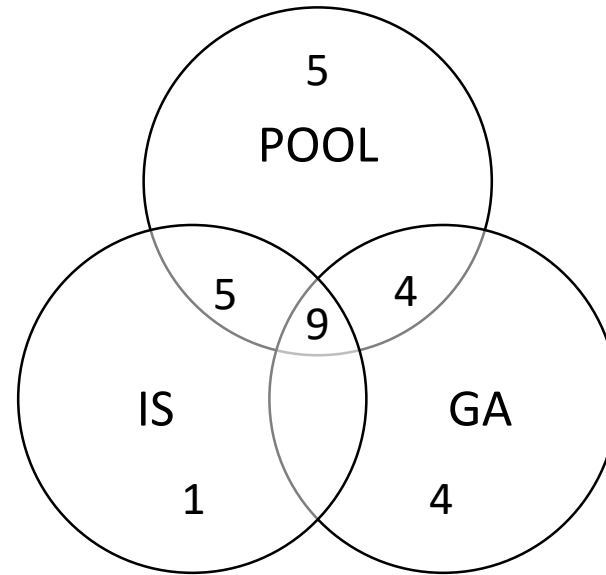
IQR: inter-quartile range; WAZ: weight-for-age Z-score according to UK growth charts 1990; BCG: bacille Calmette-Guerin.

<sup>1</sup> One child had a negative HIV PCR test at 12 weeks of age, 3 months before enrolment and an unsuccessful HIV ELISA test (specimen leaked) at enrolment which was not repeated as the child withdrew from the study before the first follow up visit; <sup>2</sup> Written record in immunisation card or visible BCG scar in right deltoid area; <sup>3</sup> During the study period, there was a global stock-out of tuberculin which resulted in a number of participants not having the test.

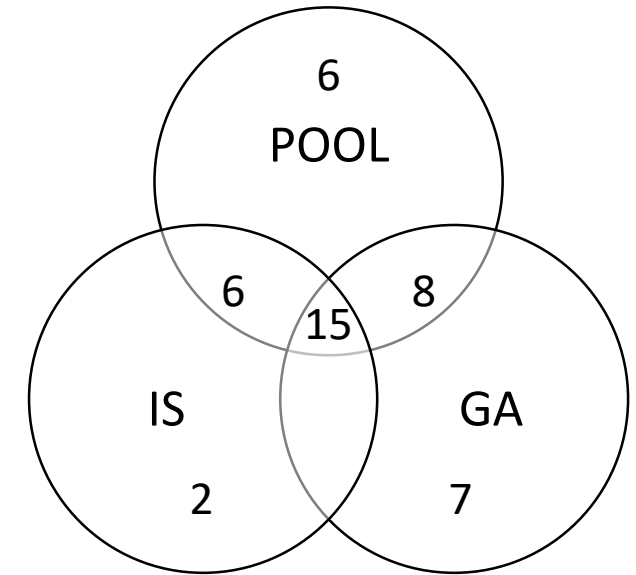
a. Culture results only



b. Xpert results only



a. Culture and Xpert results combined



SDC7. Figure 4. Incremental yield of different specimen types by culture, Xpert MTB/RIF and culture/Xpert MTB/RIF combined. GA: gastric aspirate; IS: induced sputum.

Footnote to Figure 3 (see figure legend in main article text and .TIFF image):

GA: gastric aspirate; IS: induced sputum.

<sup>1</sup>NPA specimens are not included as they had no incremental yield (i.e. all children positive by Xpert or culture on NPA were also detected by one of the other specimen/test combinations).

Percentages reflect the number of children who were confirmed by the relevant specimen/test combination as a proportion of the total number of children confirmed by any specimen type using the specified test (Xpert [4a]/culture [4b]/Xpert and culture combined [4c]). 4a: N=41 reflects 41 children who were culture-confirmed on any specimen type; 4b: N=28 reflects a total of 28 children who were Xpert-positive on any specimen type; 4c: N=44 reflects a total of 44 children who were confirmed on culture or Xpert on any specimen type.

## Chapter 10

### Discussion

Current methods to confirm the diagnosis of PTB, the most common form of TB in children, rely on relatively invasive procedures for the collection of respiratory specimens, are insufficiently sensitive and too resource-intensive for implementation in the majority of settings where children with TB live. Currently, most childhood PTB remains undetected and under-reported, despite the high morbidity and mortality posed by PTB in children living in settings with the highest burden of TB.

There has been increasing acknowledgement globally that children bear a disproportionate burden of TB, a disease that is readily preventable and treatable, and that greater efforts must be placed to address the many gaps that contribute to and perpetuate the current situation <sup>193,243</sup>. For the first time, the WHO included estimates of the burden of TB in children in its annual global TB report in 2012. In 2017, WHO estimated the global burden of TB in children below the age of 15 years to be over one million <sup>2</sup>. Efforts to estimate TB incidence and mortality using modelling approaches have provided additional insights into the burden of paediatric TB <sup>3,42,244</sup>. The WHO “End TB strategy” <sup>193</sup> and “Roadmap for childhood tuberculosis” <sup>243</sup> identified two pressing needs to improve the diagnosis of PTB in children: 1) the evaluation of more feasible approaches for implementation in high TB-burden settings using available tools, and 2) the development of new diagnostic assays with

better sensitivity than currently available methods. The development of novel diagnostic tests specifically tailored for children who mostly have paucibacillary disease has been slow due to the practical and methodological challenges of designing diagnostic studies which consider the complexity of paediatric TB and the specific needs of children <sup>31,61,174,245</sup>.

## **10.1 Relevance of standard rigorous approaches to paediatric PTB diagnostic studies and unique contributions of this cohort**

Increasingly, guidelines have been proposed for more standard approaches to diagnostic research for paediatric PTB <sup>31,61,174</sup>. These guidelines call for diagnostic studies to include symptomatic children from representative demographic and clinical groups (specifically including young and HIV-infected children), based on well-defined entry criteria, and for standard procedures to be followed for both the investigation and the reporting of results. In keeping with these recommendations, I recruited a large hospital-based cohort of 608 children presenting with suspected PTB based on clinical and epidemiological characteristics, from a setting with high burden of TB and HIV. The aims of this research were to create a rigorous framework for evaluating novel diagnostic strategies for the diagnosis of paediatric PTB, including future work on diagnostic biomarkers, and to evaluate a number of diagnostic strategies to detect *M.tb* which could be more feasible to implement in resource-limited settings.

In this cohort, all relevant age groups were well represented, with a particularly high proportion (14%) of young infants below 6 months of age. Children

>5 years of age comprised 10% of the cohort: this is probably a result of older children mostly being diagnosed at the primary health care level, as the diagnosis of PTB in this group is easier, given more specific symptoms and older children's ability to produce sputum. The cohort also included a representative proportion of HIV-infected children (n=72). Children were enrolled using well-defined symptoms of chronic duration, but children with acute cough were also included, consistent with emerging data suggesting that the presentation of PTB in young children is frequently not typical <sup>45,48,49,246</sup>. All study aspects required for the classification of children into the proposed clinical case definitions for PTB in children <sup>174</sup> were completed using SOPs, including laboratory aspects for specimen collection, transport and processing. All SOPs are provided as appendices to this dissertation. Details on the procedures followed were also made available in all the published articles to date which have resulted from this dissertation.

Using the recruitment strategy outlined above and presented in detail in chapter 3, a diagnosis of PTB was made in approximately half of the cohort. This demonstrates that using clearly defined entry criteria is useful in recruiting a cohort where the prevalence of PTB is substantial, and provides an efficient design when needing to evaluate new diagnostic tools. At the same time, half of the children enrolled were not diagnosed with TB, but presented with similar symptoms and clinical features. These children provide an important control group to study non-TB conditions with which children from high-TB burden setting may also frequently present.



High cohort retention to month 2 (92%) and month 6 (87%) was achieved, and allowed for verification of the diagnosis in the majority of cases at the month 2 follow-up. In addition, the rich longitudinal data collected will be analysed in future, and will contribute important data to characterize the clinical response to antituberculosis treatment in TB cases and progress in non-TB cases (symptomatic controls). Response to treatment is one of the diagnostic criteria proposed by the NIH consensus clinical case definitions for PTB in children, and it is an aspect that was identified as needing more empirical evidence (174).

Several important and some unexpected observations were made during the analysis of this cohort. Approximately 80% of children across most age groups (except infants <6 months of age) and other clinically relevant groups (e.g. TB cases vs. symptomatic controls, HIV-infected vs. -uninfected children) had well-defined symptoms: as this was the main entry criterion, it strengthens the validity of the data. Conversely, approximately 20% across all clinical groups had a non-specific clinical presentation. Of interest is that a shorter duration of cough was equally prevalent across all age groups and HIV status, including in the youngest infants. In infants <6 months of age, the higher proportion with non-typical symptoms was rather due to significantly lower prevalence of failure to thrive and of poor feeding. This is not in contrast with recent reports of TB presenting as acute pneumonia or with short history of cough in infants <sup>45,48,246</sup>: indeed, I showed that the majority of children (>50%) regardless of age did not report persistent unremitting cough >14 days, even in the group who had microbiologically confirmed PTB. This study was not designed to analyse the value of symptoms for the diagnosis of PTB in children, but it does

highlight the need to study the non-typical presentation of PTB in children in greater depth. A better understanding of the pathophysiology of such presentations, in relation to age, immunological status and severity of disease, would be useful for developing targeted diagnostic assays and diagnostic strategies aimed at detecting TB in this patient group. The lack of poor feeding and poor growth in the youngest infants likely points to the fulminant progression of TB in young infants, and emphasizes the need for sensitive assays that are able to detect early TB disease in this vulnerable group of children, who are at high risk of death <sup>42</sup> and poor outcomes <sup>247,248</sup>.

As a diagnostic tool, chest radiography had several limitations, as has been noted to some degree in the literature. Firstly, the radiological findings are reported from a consensus process involving a minimum of two highly experienced readers. Even with skilled readers, the variability was high and required a third reader in many instances. Inter-reader variability is a well-known challenge when interpreting paediatric films <sup>93,218</sup>, especially for the detection of intrathoracic lymph nodes <sup>200,249</sup>, which are the first and most frequent manifestation of PTB in children. It is expected that in routine clinical care, recognition of radiological patterns of PTB may be less reliable <sup>250,251</sup>. Training clinicians to read the CXRs of children with suspected TB has had limited success <sup>179</sup> but should be further improved. The usefulness of this tool is further reduced by the limited access to chest radiographs and radiologists in low-income countries <sup>252,253</sup>. As a strength, this study was carried out at a tertiary hospital, with access to specialist services, laboratory and imaging. Although a film with features typical of PTB was present in 13% of symptomatic controls, I was able to

collect sufficient data to describe the spectrum of non-TB disease in a representative proportion of these children. This will be analysed in detail in future for better characterisation of non-TB conditions and pathogens which radiologically may mimic TB. Another aspect which will be the focus of future work, is PTB presenting with non-typical radiological features. This has been described in a recent cohort of HIV-infected children <sup>254</sup>, but requires further detailed analysis for HIV-uninfected children of all ages.

Chest radiography was useful, however, to characterise the spectrum and severity of PTB. I could show that severe TB disease correlated with higher bacillary load, with 76% of children with radiologically severe PTB being microbiologically confirmed vs. 40% of children with non-severe PTB.

## **10.2 Bacteriological yield achieved in children with PTB and in subgroups of interest**

Of 281 (46%) children diagnosed with PTB, 117 (41.6%) were microbiologically confirmed by Xpert or culture. This high diagnostic yield is the result of careful specimen collection, including the collection of different specimen types and using different diagnostic tests. The small group of children initially misclassified as symptomatic controls included eight children with microbiologically confirmed PTB and highlights the importance of follow-up of all children in whom there is a clinical suspicion of PTB.

This is the first study documenting different bacteriological categories by bacillary load in young children with confirmed PTB. An unexpectedly high

proportion of young infants <6 months of age had severe PTB, associated with high bacillary load and a high proportion were smear-positive. Although smear positivity has been documented in infants before <sup>27,71</sup>, the detailed bacteriological status which I have documented in infants adds depth and emphasizes the urgent need to develop diagnostic tests that can rapidly confirm and quantify *M.tb* in children, including in the youngest infants, who are frequently perceived to not pose an infection risk. This finding also has major implications for the aggressive treatment of young infants, and for stricter infection control measures to be followed when managing and investigating children including infants with suspected PTB. These data also support the selection of participants in an ongoing clinical trial of antituberculosis treatment shortening for paediatric DS-TB, using the WHO-recommended first line drug regimen at revised 2010 higher dosages (e.g. the SHINE trial, ISRCTN63579542), which compares the efficacy and safety of the standard 6 months vs. 4 months antituberculosis therapy in children with non-severe DS-TB. Children are eligible to enter the SHINE trial if they have non-severe and sputum smear-negative PTB. Data from my study support this selection, and indicate that greater caution should be exercised when planning treatment shortening regimens specifically for infants. Consideration should be given to recommending a 4-drug regimen for DS-TB in infants, the same as what is currently recommended for smear-positive/severe PTB and HIV-associated PTB, and routinely in adults. Future priority should be given to the analysis of treatment response in this patient group, in order to inform decisions around the most appropriate antituberculosis regimens for young infants.

HIV-infected children were identified as a second high-risk group, with disseminated and extrapulmonary TB both being more prevalent in HIV-infected than in uninfected children. Poor uptake of ART and poor viral control were common in HIV-infected children in this cohort, showing that more efforts are needed to ensure their early HIV diagnosis, treatment and retention in care. The significantly higher proportion of HIV-infected children who were prescribed empirical antituberculosis treatment without microbiological confirmation confirms that the diagnosis of PTB is particularly challenging in the presence of HIV-coinfection, and new diagnostic tools should also prioritise the detection of *M.tb* in HIV-infected children. Rapid detection is particularly relevant to this group as well, given recent published evidence that any diagnostic and treatment delay is associated with increased risk of death in HIV-infected children with advanced immune suppression <sup>21</sup>.

Unexpectedly, isoniazid monoresistant TB was associated with high bacillary load and smear-positivity. There may be underlying epidemiological factors for the advanced disease presentation observed in children with isoniazid monoresistant PTB. Given recent studies reporting poorer outcomes in adults and children <sup>222,224,255</sup> with this form of DR-TB, diagnostic assays which include testing for isoniazid susceptibility are clearly needed.

### 10.3 Key findings from individual diagnostic sub-studies

I performed an extensive evaluation of stool as a more feasible specimen for the diagnosis of PTB in children from a resource-limited setting. Using different stool processing methods, I was able to show that Xpert on stool could rapidly confirm PTB in children presenting with radiological features suggestive of severe PTB. I also evaluated a novel centrifugation-free processing method which showed similar results, also using a simple swab. This approach will be tested in future using more sensitive assays such as Xpert Ultra<sup>256,257</sup>. In critically ill children, stool collection may be a safer alternative to invasive sputum collection procedures and should be considered as a potentially useful diagnostic strategy. I also showed that a second stool test using Xpert resulted in improved *M.tb* detection and was very effective in reducing the proportion of non-determinate results. As a limitation, the diagnostic sensitivity of stool testing by Xpert, even with two tests, was significantly lower than the diagnostic yield of Xpert or culture on respiratory specimens. Furthermore, the low bacillary concentrations in stool specimens resulted in a high proportion of indeterminate rifampicin resistance results on Xpert, thereby limiting the utility of stool for the detection of simultaneous rifampicin resistance.

I found that stool culture had limited utility to detect *M.tb*, due to the high proportion of bacterial/fungal culture contamination observed in stool specimens using standard decontamination protocols for mycobacterial liquid culture. Laboratory experiments to reduce stool culture contamination should precede further clinical evaluation of stool culture to detect *M.tb* in children.

Finally, I showed that pooling multiple respiratory specimens for Xpert and culture did not have substantial diagnostic benefit over testing a single GA, and I propose that GAs may in fact be considered to be naturally pooled specimens. The collection of high quality, high volume GAs may have contributed to the high diagnostic utility of this specimen type and underscores the importance of the quality of specimens for diagnostic testing. The study nurses and clinicians provided regular training to hospital nurses on the appropriate collection of GAs and other respiratory specimens, and many of the GAs tested as part of this study were collected by hospital nurses, further emphasizing its feasibility in routine care.

In all my diagnostic analyses, I accounted for all missing specimens and results, and reported the full spectrum of diagnostic test results, including non-evaluable results, contaminated cultures and discrepancies between tests. These are important considerations for understanding the true utility of diagnostic tests, and are aspects that are not frequently reported or discussed in paediatric TB diagnostic studies.

## **10.4 Limitations**

This large diagnostic cohort study was implemented in a routine clinical environment, at a tertiary and a district-level hospital. The study population is therefore representative of the paediatric patient population who is typically referred to hospital for investigation in a high-TB burden setting. Although it may be argued that these findings are not generalisable to all clinical settings, e.g. children who are managed at primary care clinics only, the aim of the study was to specifically target

young children, who face the highest burden of TB disease, and in whom PTB is most difficult to confirm bacteriologically and who are also a high risk group for disease progression and poor outcomes if undiagnosed. The hospital setting also enabled the children enrolled in the study to be thoroughly investigated, which resulted both in a substantial number having bacteriological confirmation of *M.tb*, and also in many of the symptomatic controls having an established alternative diagnosis. The hospital setting was challenging for follow-up, as the majority of children did not reside in the immediate vicinity, and many resources had to be allocated to participant tracing. However, a high retention of 87% to 6 months was achieved despite the non-interventional nature of this observational cohort study. Because the study personnel were not responsible for the clinical decision to treat or administering antituberculosis treatment, frequent contact with the relevant local clinics had to be made to ascertain issues around treatment regimens and dosages, and to assess adherence.

The study has several other limitations typically associated with the diagnosis of paediatric PTB, including imperfect and, at times, unavailable study measures. During the study period, South Africa experienced a stock-out of Mantoux skin tests, resulting in a proportion of children not having had a test of infection, as IGRAs were not available to the study. CXRs were at times of unacceptable quality and a few were not available at the time when independent dual reading was done. Some data which was initially planned for analysis was difficult to obtain, specifically information around previous or recent isoniazid preventive therapy and antibiotic use before enrolment. Finally, although the study had an established schedule for specimen



collection, this was not always achievable, due to changing clinical condition, or discharge from hospital before specimen collection could be completed. Despite some children not having all the data required, the majority of children could be successfully classified into the NIH clinical case definitions because of the rigorous follow-up implemented, which included follow-up clinical evaluation, chest radiography for all children and additional specimen collection for microbiological testing in all TB cases. These long-term follow-up data, and the complete categorisation of children into the proposed research clinical case definitions for paediatric PTB are not presented in this dissertation, but are planned for future analysis to evaluate follow-up as a diagnostic tool, as proposed by the NIH consensus documents for diagnostic research in paediatric PTB.

## **10.5 Impact on policy and practice**

I identified young infants <6 months of age as a potentially highly infectious patient group and with significant burden of severe PTB. Healthcare facilities need to implement strict infection control measures when managing infants with suspected TB to prevent nosocomial spread. Infection control measures should also be implemented when managing Xpert-positive children with PTB, as one in four children is likely to be smear-positive. Although infants have less tussive force than older children and adults, secretions are aerosolised during sputum collection procedures. In addition, infectiousness is not only determined by bacillary concentrations in sputum, and prolonged low-grade exposures (for example, to

coughing infants) can also lead to infection of anyone who may be in close or continuous contact with these infants.

Aggressive contact management and provision of preventive therapy to HIV-infected pregnant women to prevent TB in young infants should be priority given the high risk of disease progression. Antituberculosis treatment regimens for infants should carefully consider the spectrum of TB disease and the ability of the young infant to contain the organism.

I further identified HIV co-infected children as a particularly vulnerable patient group despite current policies for early HIV testing and initiation of ART, with many HIV-infected children not on ART. HIV-infected children need continued efforts to enforce early HIV testing, early access to ART and retention in care. Investigation for a TB remains an important opportunity to do so.

For the detection of *M.tb*, pooling respiratory specimens was not effective in substantially increasing the diagnostic yield of *M.tb*, and had similar detection as a single GA. Pooling is therefore not recommended as a diagnostic strategy in young children with suspected PTB. Gastric aspirates could be considered naturally pooled specimens and have a high yield when tested by culture and Xpert if high volume and high-quality specimens are collected. Adequate training for healthcare personnel routinely performing gastric aspiration should be provided to maximise the diagnostic yield of this relatively simple specimen collection technique.

Furthermore, Xpert testing of stool specimens can provide a rapid result in a high proportion of children who have severe forms of PTB, but processing is currently too resource-intensive for implementation at peripheral laboratories. A novel

processing method which does not require centrifugation could be useful in such settings, but more data are needed using more sensitive assays such as Xpert Ultra. In contrast, based on results from my research, stool culture is currently not recommended for diagnosis of PTB in children.

## **10.6 Future directions**

Through this cohort, I have established a well-characterised bio-repository of specimens, including blood, urine and stool, for the evaluation of promising biomarkers of disease and treatment response. I have identified young infants as a priority group for the development and evaluation of new diagnostic tests. Having documented the rapid progression to highly infectious and disseminated forms of TB in this group, new diagnostic assays and biomarkers should be able to identify infants at risk of progression i.e. these assays should detect TB disease in its early stages. Quantitative assays measuring bacillary burden and/or TB disease severity should be specifically prioritised, and evaluated for their potential to also measure response to treatment. Biomarkers for treatment response would be particularly useful to inform appropriate treatment regimens for children, including young infants who may not be an appropriate group to target for shortened regimens given the severity of PTB disease observed and their developing immune system.

Diagnostic tests able to distinguish TB from other chronic infectious and inflammatory conditions common in HIV-infected children are also a priority, and would prevent unnecessary treatment.

I have, through this research, established collaborations with local and international groups., I have obtained funding to specifically address these questions, and plan to evaluate a promising serum biosignature for diagnosis and treatment response in this and new prospective paediatric cohorts<sup>258</sup> as well as urine proteomic signatures.

Planned future work for this cohort includes:

- Investigation of selected serum and urine biosignatures as markers of diagnosis and treatment response using bio-banked specimens. The proposed serum bio-signature uses nanodisc technology to detect two *M.tb*-derived molecules, peptides of ESAT-6 and CFP-10, and one host-derived immune cytokine, IP-10, to detect active TB disease and monitor treatment response. Preliminary data on adults with paucibacillary disease are encouraging and suggest that the signature has high sensitivity and specificity for *M.tb* and is not detected in appreciable concentrations in latent TB infection<sup>258,259</sup>. This work is in collaboration with Arizona State University, Arizona, USA.
- For urine-based diagnosis, exploratory work involves a proteomics approach to develop a bio-signature of abundant proteins associated with active TB vs. TB infection and non-TB state in children with suspected TB. This work is in collaboration with Boston Children's Hospital and Harvard University, MA, USA.
- Characterisation of long-term follow-up as a diagnostic tool for paediatric PTB;

- Characterisation of the full spectrum of alternative diagnoses, including additional pathogens detected, in the symptomatic control group;
- Analysis of short- and long-term treatment response using clinical, radiological and bacteriological data (all collected and ready for analysis), including analysis of potential markers of antituberculosis response to treatment;
- Methodological description and analysis of chest radiology, including detailed characterisation of all typical and non-typical films from both cases and controls.

This cohort has also supported a number of sub-studies, including a pilot study of the respiratory viral and bacterial pathogens associated with TB cases and controls, and an evaluation of the impact of such co-infections on the presentation and treatment response in children with TB; and a study on lung function in both TB cases and controls, at diagnosis and longitudinally to assess the effect of *M.tb* and other common respiratory pathogens on lung health in children.

## **10.7 Conclusions**

It is evident that current tools are inadequate to diagnose PTB in children, and that more investment should be made to develop and evaluate assays that can detect TB disease in its early stages, especially in high risk groups. Assays that can also be used as markers of treatment response are needed to inform the future evaluation of novel, including shorter, antituberculosis regimens for children. No such markers are currently available. In 2015, a consensus blueprint paper to address gaps in

diagnostic for paediatric TB was published <sup>260</sup>. It again called for diagnostic research for PTB in children to implement standard approaches, include representative sample populations and report findings using the consensus guidelines previously developed <sup>31,174</sup>. The document outlines the desirable qualities of potential biomarkers, including use of a non-sputum matrix, the ability to distinguish latent TB infection from active disease and TB disease from non-TB conditions, across all spectra of age and disease severity, and including all clinically relevant groups. Assays should have high sensitivity to rule out TB disease if negative, and high specificity to distinguish TB from other common childhood illnesses and non-mycobacterial infections. Adequate follow-up of all cases was highlighted as a critical element in biomarker evaluation. Already, potentially useful biosignatures are being tested on paediatric populations, including gene expression profiles <sup>261,262</sup> and immunological markers of TB disease <sup>263,264</sup>. The large cohort I have established, with its systematic and comprehensive follow-up evaluation of all children, including PTB cases and symptomatic controls, is unique and will contribute critical data to inform the value of follow-up both for diagnosis and for evaluation of treatment response, and to provide initial data on the performance of selected novel bio-signatures for diagnosis and treatment response using stored urine and serum specimens. These data will contribute towards an evidence-based framework for the future evaluation of diagnostic and prognostic biomarkers for children with PTB.

## Chapter 11

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## Appendix A

### Publications relevant to this dissertation

#### A.1 Published articles relevant to the doctoral dissertation

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### **A.3 Preliminary data on diagnostic serum biosignature for future evaluation**



# Clinical Evaluation of a Blood Assay to Diagnose Paucibacillary Tuberculosis via Bacterial Antigens

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**BACKGROUND:** The diagnosis of active tuberculosis (TB) cases primarily relies on methods that detect *Mycobacterium tuberculosis* (*Mtb*) bacilli or their DNA in patient samples (e.g., mycobacterial culture and Xpert MTB/RIF assays), but these tests have low clinical sensitivity for patients with paucibacillary TB disease. Our goal was to evaluate the clinical performance of a newly developed assay that can rapidly diagnose active TB cases by direct detection of *Mtb*-derived antigens in patients' blood samples.

**METHODS:** Nanoparticle (NanoDisk)-enriched peptides derived from the *Mtb* virulence factors CFP-10 (10-kDa culture factor protein) and ESAT-6 (6-kDa early secretory antigenic target) were analyzed by high-throughput mass spectrometry (MS). Serum from 294 prospectively enrolled Chinese adults were analyzed with this NanoDisk-MS method to evaluate the performance of direct serum *Mtb* antigen measurement as a means for rapid diagnosis of active TB cases.

**RESULTS:** NanoDisk-MS diagnosed 174 (88.3%) of the study's TB cases, with 95.8% clinical specificity, and with 91.6% and 85.3% clinical sensitivity for culture-positive and culture-negative TB cases, respectively. NanoDisk-MS also exhibited 88% clinical sensitivity for pulmonary and 90% for extrapulmonary TB, exceeding the diagnostic performance of mycobacterial culture for these cases.

**CONCLUSIONS:** Direct detection and quantification of serum *Mtb* antigens by NanoDisk-MS can rapidly and accurately diagnose active TB in adults, independent of disease site or culture status, and outperform *Mycobacterium*-based TB diagnostics.

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Tuberculosis (TB)<sup>10</sup> is a highly prevalent and deadly infectious disease, with a worldwide incidence of more than 10.4 million new cases and 1.4 million deaths in 2015 according to the latest World Health Organization estimates (1). In China and other developing countries, TB diagnosis still heavily relies on microbiologic techniques, including smear microscopy for acid-fast bacilli (AFB) and the "gold standard" of mycobacterial culture (2, 3), which has a very long sample-to-answer timeframe. Both methods, however, have low clinical sensitivity for paucibacillary TB cases, which account for >60% of new TB cases each year in emerging TB-endemic areas such as China (1, 4, 5). The PCR-based Xpert MTB/RIF assay can increase the diagnosis speed and clinical sensitivity in multibacillary TB, but it still requires sputum or invasive biopsy specimens, exhibits moderate clinical sensitivity in patients with paucibacillary TB (e.g., *Mycobacterium tuberculosis* (*Mtb*) culture- or smear-negative TB cases), and cannot differentiate between live and nonviable *Mtb* bacilli to monitor response to anti-TB therapy (6–10). Blood-based IFN- $\gamma$  release assays measure ex vivo immune responses to assay-introduced *Mtb* antigens and cannot distinguish between active TB and latent TB infection (11, 12).

*Mtb* bacilli robustly secrete CFP-10 (10-kDa culture filtrate antigen) and ESAT-6 (6-kDa early secretory antigenic target) to promote immunopathologic responses, and loss of either gene results in a significant reduction in virulence, strongly indicating these factors are specific for virulent mycobacteria (13–16). These factors appear to represent ideal biomarkers for active TB because their detection in any clinical patient sample constitutes evidence of active TB disease (17, 18). Current *Mtb* antigen immunoassays have poor diagnostic performance, however, which appears to result in part from antigen masking by host

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<sup>10</sup> Nonstandard abbreviations: TB, tuberculosis; AFB, acid-fast bacilli; *Mtb*, *Mycobacterium tuberculosis*; NTM, nontuberculous mycobacteria; MS, mass spectrometry.

antibodies as well as potential interference by homologous antigens secreted by nontuberculous mycobacteria (NTM). We recently developed a peptide-based approach that overcomes both these issues by using antibody-conjugated porous discoidal nanoparticles (NanoDisks) to (a) bind *Mtb*-specific CFP-10 and ESAT-6 peptides present in digested serum samples of TB cases and (b) enhance their quantitative detection by bench-top MALDI-TOF mass spectrometry (MS) (19). In this study, we employed an adult Chinese patient population to validate whether NanoDisk-MS assay results correlated with clinical diagnoses, outperformed culture and histology tests used to diagnose active TB in adults, and revealed differential clinical sensitivity for pulmonary and extrapulmonary TB cases or *Mtb* culture-positive and culture-negative TB cases.

## Methods

### PARTICIPANTS

A total of 376 adults ( $\geq 18$  years of age) who visited the Shandong Chest Hospital in China for TB screening in 2013 were enrolled in this study. Eligibility criteria for participants were no history of prior TB treatment or HIV infection and written informed consent. Exclusion criteria for eligible subjects were missing or contaminated samples, incomplete procedures (lost to follow-up), and/or diagnosis of NTM infection. Study approvals were obtained from the Institutional Review Boards at Houston Methodist Hospital and Shandong Chest Hospital. Standard bacteriological and radiological tests were performed by an ISO15189-certified TB reference laboratory at Shandong Chest Hospital. Clinical services for sample and information collection were performed under contract on a fee-for-service basis.

### CHARACTERIZATION OF PARTICIPANTS AND DEFINITIONS

Demographics, risk factors, clinical history, and findings were documented on a standardized case report form. The Diagnostic Criteria for Tuberculosis (WS288–2008) guidelines established by the People's Republic of China were used as a general guideline to diagnose and classify all patients. Enrolled study participants were evaluated for risk of active TB based on their chest radiography, symptoms, and medical history results. Individuals suspected to have active TB disease based on one or more of these criteria were evaluated with AFB smears of 3 consecutive early morning sputa samples ( $\geq 24$  h apart) and 8 weeks of mycobacterial cultures of sputum (3–5 mL) or specimens from other suspected infection sites. The Xpert MTB/RIF assay was not routinely available at the study site during this study. All enrolled participants had a 10-mL blood sample drawn before the start of anti-TB therapy. Venous blood samples were drawn into red top Vacutainer Tubes, which were gently inverted 5

times and incubated at room temperature for 30 min; then, they were centrifuged at 1000g for 10 min to isolate serum samples that were stored at  $-80$  °C until thawed for analysis. Sputum samples were immediately processed and refrigerated within 4 h of collection. Decontamination of sputum specimens followed standard internationally recommended sodium hydroxide and N-acetyl-L-cysteine (NaOH-NALC) methodology (20, 21). Serum samples were not fixed or otherwise processed before digestion, and all sample preparation and handling steps were therefore conducted in a biosafety hood designated for this study in accordance with standard biosafety protocol for handling unfixed human blood samples. Experimental details are described in the Materials section of the Supplemental Information that accompanies the online version of this article at <http://www.clinchem.org/content/vol64/issue5>.

Patients in whom the sites of disease were exclusively confined to lungs, pleura, and intrathoracic lymph nodes were classified as pulmonary TB. Patients whose disease extended to organs or tissues outside the thorax were considered to have extrapulmonary TB. Patients with both pulmonary and extrapulmonary disease findings were classified as pulmonary TB. A culture-positive TB case was defined as a mycobacterial culture-positive result, confirmed by *Mtb*-specific PCR amplification. A culture-negative TB case was diagnosed based on the physician's clinical assessment and the patient's response to treatment at the end of anti-TB therapy. Non-TB individuals were defined by: (a) negative chest radiography results and the absence of symptoms or history consistent with TB infection at the time of sample collection and follow-ups and/or (b) no anti-TB therapy administered, based on physician's clinical recommendation.

### NANODISK-MS ASSAYS

Serum samples (100  $\mu$ L) were digested for 20 min with 10- $\mu$ g sequencing-grade modified trypsin under microwave irradiation. NanoDisk particles functionalized with peptide-specific antibody were incubated with digested serum samples for 2 h at 25 °C with constant rotary mixing, after which peptide-loaded NanoDisk particles were directly spotted on the target for MALDI-TOF MS detection (19). Standard recombinant CFP-10 and ESAT-6 were trypsin-digested and analyzed on an AXIMA Resonance MALDI-TOF MS to select monovalently charged target peptide ions. CFP-10 and ESAT-6 peptide ions ( $m/z$  1593.75 and 1900.95, respectively) were selected for diagnostic NanoDisk-MS assays owing to their high signal-to-noise ratios. Isotope-labeled versions of these 2 peptides with 10-Da mass increases ( $m/z$  1603.60 and 1910.80) were synthesized as internal standards, and the MS intensity ratio of each target peptide and its internal standard in each sample was calculated for absolute quantification of serum CFP-10 and ESAT-6 concentration. Pooled human serum from non-TB cases



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was supplemented with different concentrations of CFP-10, ESAT-6, and internal standards and were processed with the aforementioned protocol to establish a standard curve (19). Each clinical sample was analyzed in 3 replicate experiments, and the mean intensity ratio value for each target peptide was used to calculate the serum concentration of its corresponding *Mtb* biomarker antigen (see Materials section in the online Data Supplement for details).

### ANALYTICAL PERFORMANCE

Clinical samples were pooled to generate a pooled “disease sample” ( $n = 20$ ) and a pooled “healthy control” sample ( $n = 200$ ) to assess the precision of the NanoDisk-MS assay. Five replicates of each of these samples were analyzed once a day for 5 days to determine intraassay and interassay precision, and the means of the 25 CFP-10 and ESAT-6 results for the pooled disease and control samples were used as reference concentrations for subsequent analytical evaluations. Limits of detection and quantification were determined as the lowest concentration that produced CFP-10 and ESAT-6 mass spectra peaks with signal-to-noise ratios  $\geq 3$  and  $\geq 5$ , respectively, after serially diluting ( $2\times$ ) disease sample with analyte-free serum from a healthy donor. These samples were analyzed for stability effects after 30 days at  $-80^\circ\text{C}$ , after incubation at room temperature ( $20\text{--}24^\circ\text{C}$ ) for 4 h, after refrigeration ( $4\text{--}8^\circ\text{C}$ ) for 24 h, and after 2 freeze-thaw cycles. Sample interference effects were assessed by analyzing CFP-10 and ESAT-6 concentrations in disease sample aliquots supplemented with different levels of clinical interferents (Assurance Interference Test Kit, Sun Diagnostics) and by comparing these values to their respective reference concentrations (22) (see Materials section in the online Data Supplement for details).

### DATA ANALYSIS

A NanoDisk-MS result indicative of active TB disease was defined as a positive signal for either CFP-10 or ESAT-6 target peptide above the limit of quantification (19). We therefore defined the NanoDisk-MS readout as the combined CFP-10 and ESAT-6 concentration in a serum sample. Definition for positive and negative results were established before the study team was unblinded to the patients' information. NanoDisk-MS test results were compared to AFB smear and mycobacterial culture results for all cases and for pulmonary and extrapulmonary TB cases. NanoDisk-MS clinical sensitivity was also compared to evaluate assay performance between patients with positive and negative AFB smear or mycobacterial culture status. Differences between groups were tested for significance with the 2-sample  $\chi^2$  test for binary variables and nonparametric Wilcoxon rank-sum tests for continuous variables, as normal distributions were not confirmed in these samples. GraphPad Prism

software (v 5.0) was used for all statistical analysis and  $P$  values  $<0.05$  were considered statistically significant.

## Results

### STUDY POPULATION

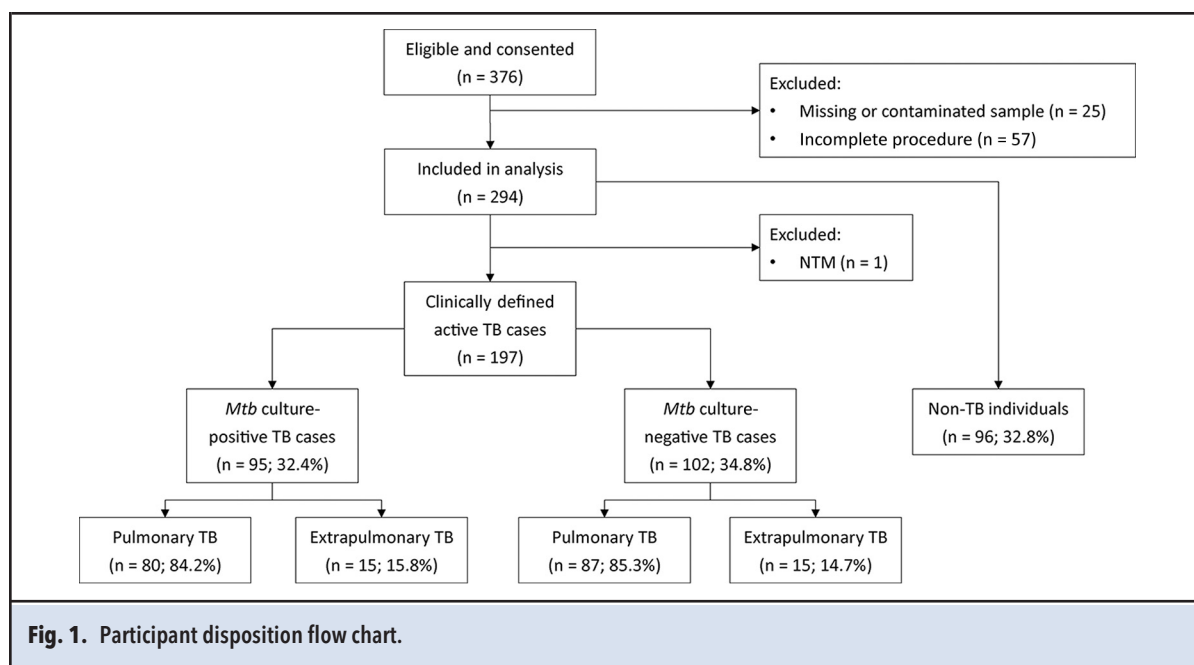
From a population of 376 individuals meeting study criteria, samples from 198 patients with active TB and 96 individuals without active TB were analyzed by our NanoDisk-MS assay (Fig. 1). Among patients with active TB, 95 had positive culture results (80 pulmonary TB and 15 extrapulmonary TB) and 102 had negative culture results (87 pulmonary TB and 15 extrapulmonary TB). One participant who was initially classified as an active TB case was later excluded from the study after analyses revealed that he was infected with an NTM. TB cases and non-TB cases did not differ by median age or gender distribution (Table 1; 44 vs 44 years of age and 65.5% vs 69.8% male, respectively). The TB group contained 167 pulmonary (84.2%) and 30 extrapulmonary (15.2%) cases (7 tuberculous meningitis, 6 peritonitis, 5 cervical lymphadenopathy, 3 genitourinary TB, 6 pericardial TB, and 3 spinal TB). Chest x-ray results revealed findings consistent with active TB disease in 82.2% of active TB cases and differed between pulmonary and extrapulmonary cases (94.0% vs 16.7%, respectively). Mycobacterial culture was positive in 48.2% of all TB patients, and the detection rate did not differ between pulmonary (47.9%) and extrapulmonary (50.0%) TB cases. AFB smear results demonstrated extremely poor overall clinical sensitivity (8.6%) and detected none of the extrapulmonary TB cases.

### OUTPERFORMANCE OF NANODISK-MS OVER SMEAR

#### MICROSCOPY AND CULTURE IN OUR STUDY POPULATION

Current immunoassays do not accurately quantify *Mtb* antigens in blood samples owing to potential interference from host antibodies and homologous NTM proteins. However, we have previously reported that the *Mtb* CFP-10 peptide TDAATLAQEAGNFER ( $m/z$  1593.75) and the *Mtb* ESAT-6 peptide WDATATELNALQNLAR ( $m/z$  1900.95) are highly specific for *Mtb* and can be used to directly quantify *Mtb* CFP-10 and ESAT-6 concentrations by MS (19). To quantify patient *Mtb* antigen concentrations, patient serum samples were trypsin-digested, supplemented with heavy-isotope variants of the *Mtb* target peptides as internal standards, and incubated with antibody-conjugated NanoDisks to selectively enrich these peptides and to enhance MALDI ionization to increase MS analytical sensitivity (Fig. 2A).

Reference biomarker concentrations for pooled disease (CFP-10, 4.6 nmol/L and ESAT-6, 6.3 nmol/L) and control (CFP-10, 0 nmol/L and ESAT-6, 0 nmol/L) samples were determined as the mean of 25 replicates, and pooled disease sample was used for subsequent ana-



lytical validation. Intra- and interassay precision (CV) were found to be 13.1% and 14.8% for CFP-10, respectively, and 12.8% and 11.6% for ESAT-6, respectively. Dilution experiments of pooled disease sample revealed excellent CFP-10 and ESAT-6 linearity ( $R^2 > 0.99$ , see Fig. 1 in the online Data Supplement) with limits of detection of 72 and 197 pmol/L and limits of quantification of 288 and 394 pmol/L, respectively (CV < 20%). NanoDisk-MS detected 88.5%–92.2% of the expected CFP-10 and ESAT-6 values after exposing aliquots of the disease sample to common storage and handling conditions, and 87.4%–94.7% of these values after contami-

nating disease sample aliquots with common clinical interferents (Table 2).

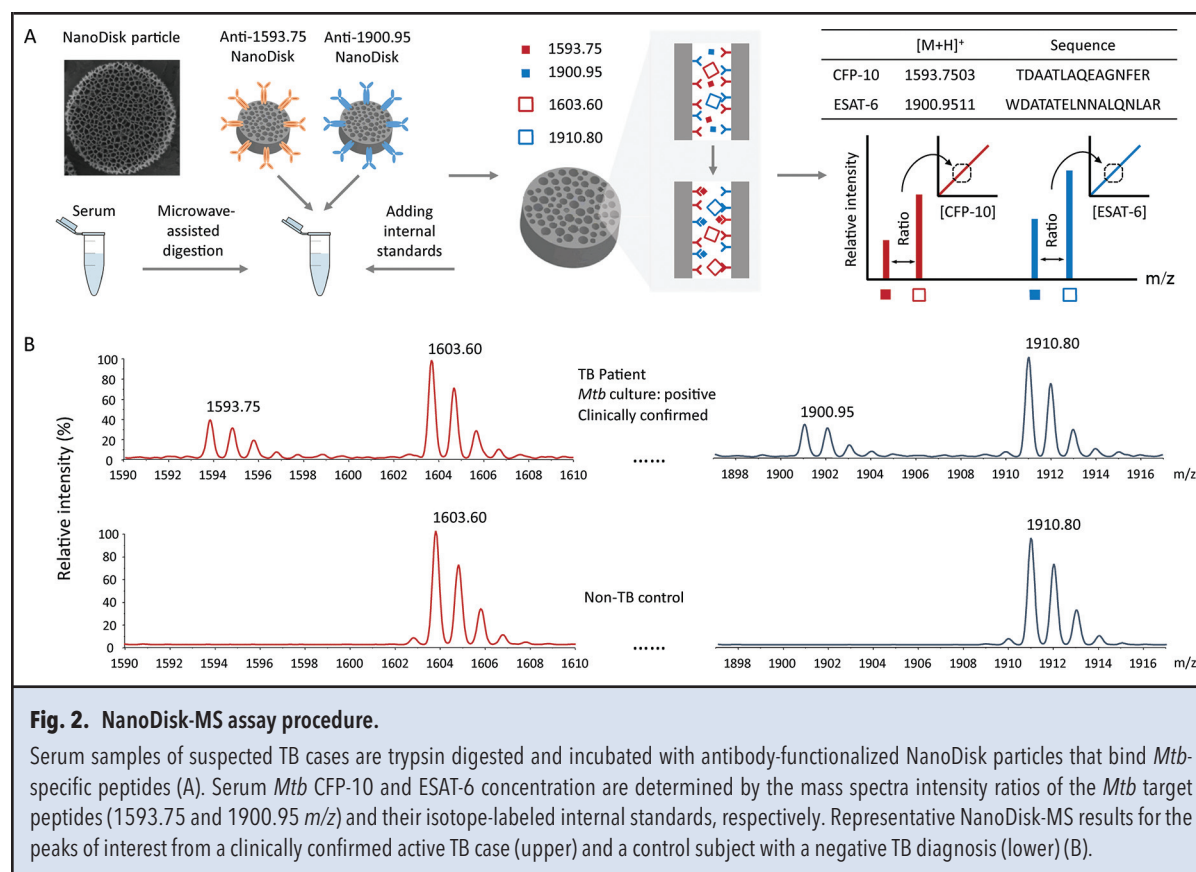
NanoDisk-MS spectra readily distinguished serum from selected individuals with and without active TB in a proof-of-principle test (Fig. 2B). Blinded analyses were thus performed on all study samples to determine the correspondence between NanoDisk-MS results and clinical TB diagnosis, with detectable *Mtb* antigen as the NanoDisk-MS criterion for active TB diagnosis. Subsequent analysis of unblinded data revealed that NanoDisk-MS detected CFP-10 and/or ESAT-6 signal in 174 of the 197 TB cases (88.3% clinical sensitivity;

**Table 1. Demographic, microbiology, and diagnostic data for study participants.**

	All TB cases (n = 197)	Pulmonary (n = 167)	Extrapulmonary (n = 30)	P value	Non-TB cases (n = 96)
Demographics					
Male (%)	129 (65.5%)	109 (65.3%)	20 (66.7%)	0.882	67 (69.8%)
Median age (IQR)	44 (26–60)	45 (27–61)	35.5 (23–57.5)	0.246	44 (30.5–59.8)
Conventional TB diagnosis					
Chest x-ray positive (%)	162 (82.2%)	157 (94.0%)	5 (16.7%)	<b>&lt;0.0001<sup>a</sup></b>	0 (0.0%)
<i>Mtb</i> culture positive (%)	95 (48.2%)	80 (47.9%)	15 (50.0%)	0.832	NA
AFB smear positive (%)	17 (8.6%)	17 (10.2%)	0 (0.0%)	0.140	NA
NanoDisk-MS (CFP-10 + ESAT-6)					
Biomarker positive (%)	174 (88.3%)	147 (88.0%)	27 (90.0%)	0.999	4 (4.2%)
Median summed biomarker conc., nmol/L (IQR)	2.567 (0.973, 4.613)	2.604 (1.033, 4.816)	1.929 (0.758, 3.531)	0.309	0 (0.000, 0.000)

<sup>a</sup> Boldface indicates significant values.

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95% CI, 83.1%–92.1%; Table 1) and 4 of the 96 study subjects without a clinical TB diagnosis (95.8% clinical specificity; 95% CI, 89.8%–98.4%; see Fig. 2 in the online Data Supplement). These results markedly outperformed mycobacterial culture (95 of 197, 48.2%; 95% CI, 41.4%–55.2%) and AFB smear (17 of 197, 8.6%; 95% CI, 5.5%–13.4%) detection rates in this population. Notably, 1 patient evaluated as TB-negative by NanoDisk-MS (ID: 50) was initially defined as a pulmonary TB case with multidrug resistance by culture and drug susceptibility testing but was reclassified as an NTM case (*M. intracellulare*) after confirmation by *groEL2* gene sequencing; the patient was excluded from the study.

#### NANODISK-MS DETECTS ACTIVE TB CASES IRRESPECTIVE OF INFECTION SITE OR CULTURE STATUS

NanoDisk-MS detected 147 of 167 (88.0%; 95% CI, 82.2%–92.1%) pulmonary and 27 of 30 (90.0%; 95% CI, 74.4%–96.5%) extrapulmonary TB cases, revealing considerably better diagnostic performance than mycobacterial culture (47.9% and 50.0%, respectively) or AFB smear (10.2% and 0.0%, respectively) results, and detecting similar median-summed *Mtb* antigen concentrations in both groups (Table 1). NanoDisk-MS also detected 87 of 95 (91.6%; 95% CI, 84.3%–95.7%)

culture-positive and 87 of 102 (85.3%; 95% CI, 77.2%–90.9%) culture-negative TB cases. Similar results were detected in study groups with AFB smear-positive and smear-negative results, in which NanoDisk-MS detected 17 of 17 (100%; 95% CI, 81.6%–100%) TB cases with positive results and 157 of 180 (87.2%; 95% CI, 81.6%–91.3%) TB cases with negative results (see Table 1 in the online Data Supplement).

NanoDisk-MS also exhibited similar clinical sensitivity for culture-positive pulmonary (73 of 80, 91.3%; 95% CI, 83.0%–95.7%) and extrapulmonary (14 of 15, 93.3%; 95% CI, 70.2–98.8%) TB cases (Fig. 3), and culture negative pulmonary (74 of 87, 85.1%; 95% CI, 76.1%–91.1%) and extrapulmonary (13 of 15, 86.7%; 95% CI, 62.1%–96.3%) TB cases diagnosed with radiological findings, symptoms, and treatment outcome results.

#### SERUM CFP-10 AND ESAT-6 CONCENTRATION DOES NOT DIFFER MARKEDLY BY SPECIFIC TB DIAGNOSIS

CFP-10 and ESAT-6 are frequently coproduced as heterodimers but were detected at different frequencies and different concentrations in active TB cases in this study (Fig. 3 and see Table 2 in the online Data Supplement). Most TB cases produced 1 or both antigens (174 of 197

**Table 2.** Analytical validation of the NanoDisk-MS assay.

	CFP-10	ESAT-6
Disease pool: <sup>a</sup>		
Mean concentration, nmol/L	4.6	6.3
Intraassay CV, %	13.1	12.8
Interassay CV, %	14.8	11.6
Limit of detection, pmol/L	72	197
Limit of quantification, pmol/L	288	394
Analyte stability after storage and handling (% untreated, mean $\pm$ SD)		
4 h @ 20–24 °C	92.1 $\pm$ 0.7	91.5 $\pm$ 1.1
24 h @ 4–8 °C	91.8 $\pm$ 0.4	92.2 $\pm$ 0.8
30 days @ –80 °C	90.7 $\pm$ 0.6	91.4 $\pm$ 0.5
2 freeze-thaw cycles	88.5 $\pm$ 0.9	89.3 $\pm$ 0.7
Influence of clinical interferences (% untreated, mean $\pm$ SD)		
Hemolysate	90.8 $\pm$ 0.5	88.6 $\pm$ 0.6
Triglycerides	94.7 $\pm$ 1.0	91.3 $\pm$ 1.0
Bilirubin	91.1 $\pm$ 0.9	88.3 $\pm$ 0.3
Abundant protein	87.4 $\pm$ 0.8	91.7 $\pm$ 0.8

<sup>a</sup> CFP-10 and ESAT-6 are undetectable in the healthy pool.

cases); however, only 40 produced both CFP-10 and ESAT-6 (20.3%), with 125 individuals producing only CFP-10 (63.5%) and 9 producing only ESAT-6 (4.6%). NanoDisk-MS readout levels (summed concentrations of CFP-10 + ESAT-6) did not differ between pulmonary and extrapulmonary cases (Table 1) but significantly differed by culture status, with modestly higher combined antigen concentrations detected in culture-negative TB patients (Fig. 4A). No such difference was found between smear-positive and smear-negative cases (Fig. 4B). Secondary analysis of serum *Mtb* antigen concentrations in pulmonary and extrapulmonary cases by culture status also did not detect group differences (Fig. 4C).

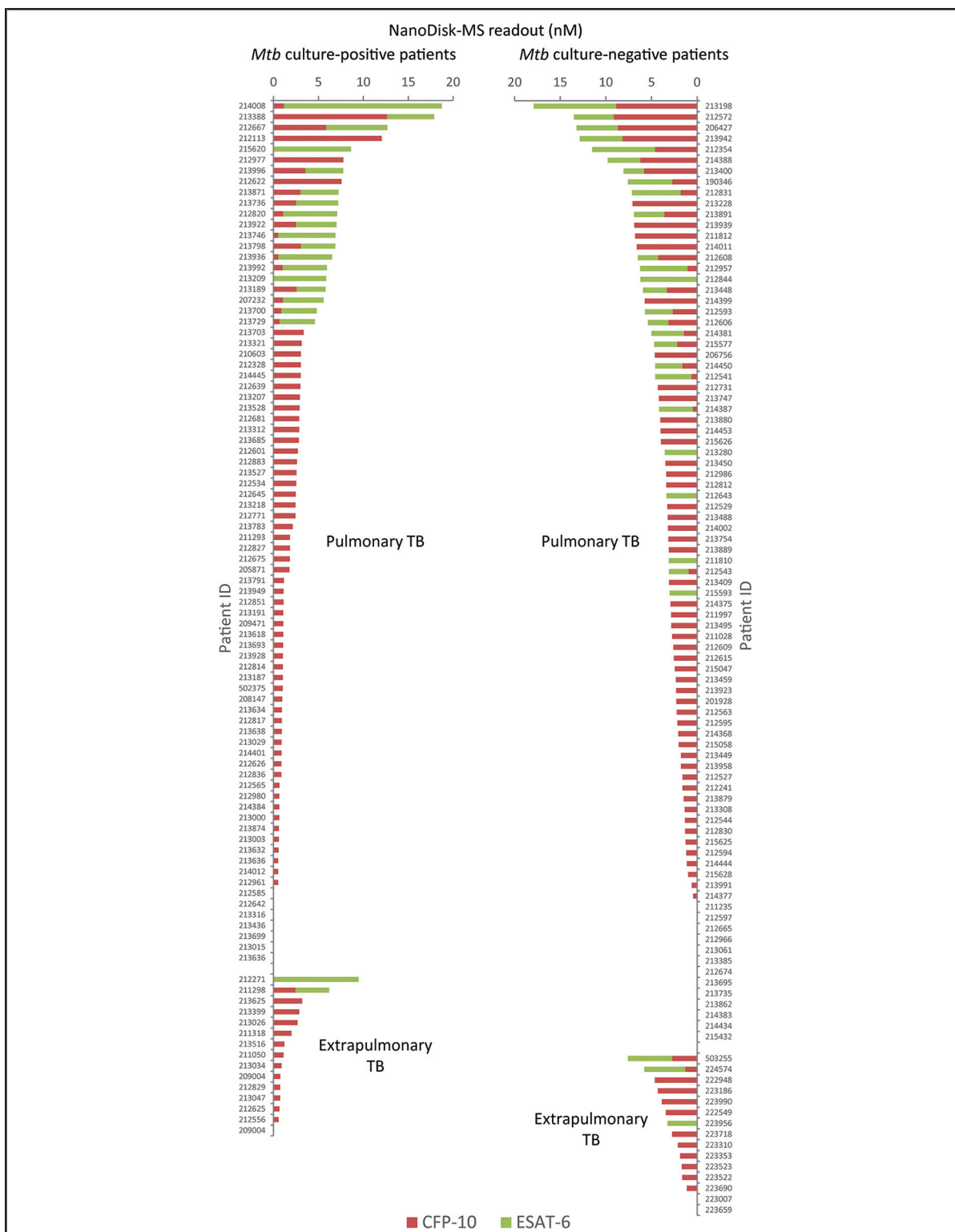
## Discussion

Rapid and reliable diagnostic assays to detect active TB cases are urgently needed for global TB control efforts. Bacteriologic (mycobacterial culture and AFB smear) and Xpert MTB/RIF TB assays commonly used for TB diagnosis worldwide require *Mtb*-rich specimens for accurate diagnosis. These methods thus exhibit suboptimal clinical sensitivity in paucibacillary patient populations, including patients with low mycobacterial loads or poor sputum production, and the majority of HIV-positive (23) and pediatric patients (24, 25). The collection of diagnostically useful samples can require invasive procedures (7) and technical variables that can influence test

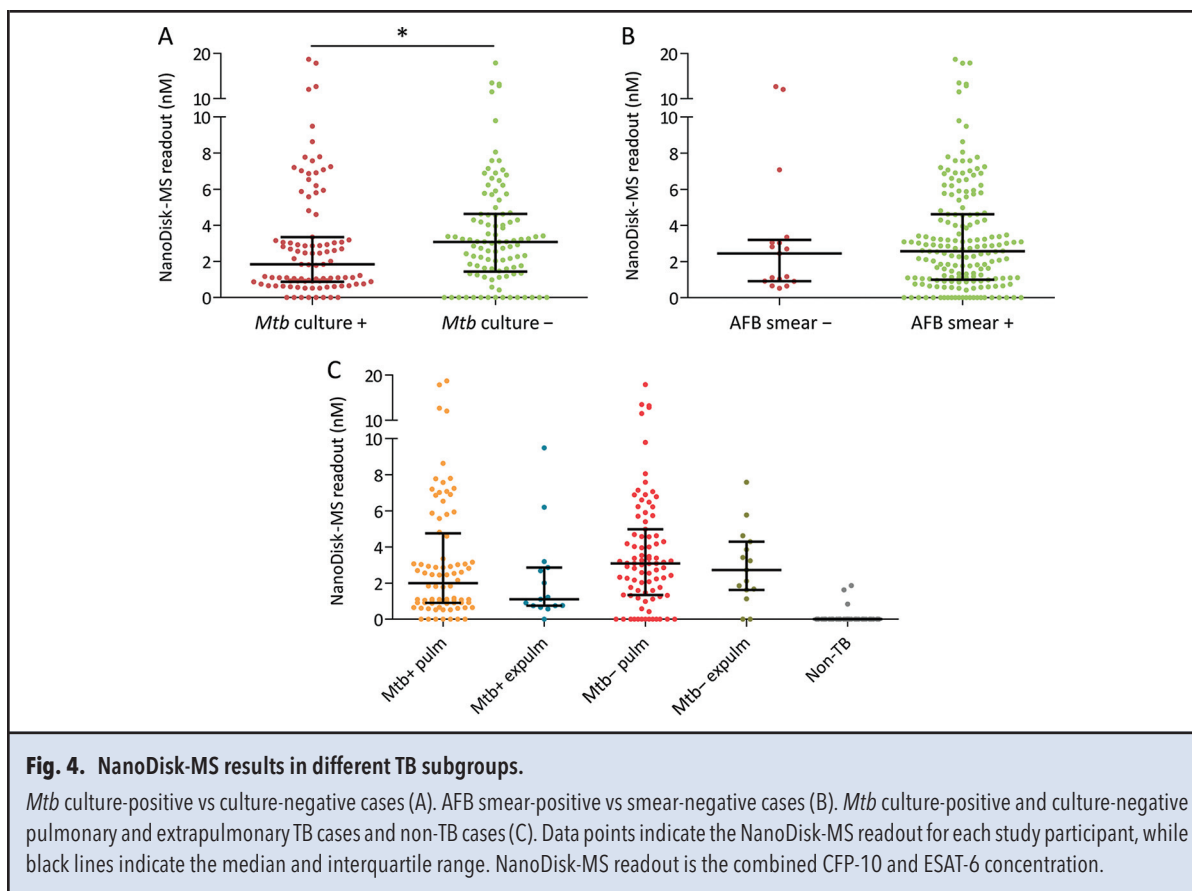
results. The *Mtb* virulence factors CFP-10 and ESAT-6 should always be detectable in active TB disease, however, regardless of the disease site or local *Mtb* concentrations. We therefore recently developed a NanoDisk-MS assay that rapidly and sensitively detects circulating *Mtb* antigens to allow robust diagnosis across the spectrum of TB cases. This assay allows for rapid TB diagnosis with several advantages over existing tests. These include (a) clinical sensitivity independent of disease site (pulmonary or extrapulmonary) or mycobacterial load (culture-negative TB cases), (b) use of 2 *Mtb* virulence factors to allow for case-to-case variation, (c) detection of serum biomarkers with low biosafety concerns due to lack of infectious aerosols, and (d) precise serum *Mtb* antigens quantification.

In our study population, NanoDisk-MS accurately detected CFP-10 and/or ESAT-6 in the serum of active TB cases, achieving 88.3% clinical sensitivity across all the disease-affected subgroups in this study, with 95.8% specificity. Patients with paucibacillary conditions that are missed by current TB tests are typically diagnosed subjectively on the basis of patient history, symptoms, and imaging results, as well as their observed response to anti-TB therapy. According to the 2015 World Health Organization Global Tuberculosis Report (1), mycobacterial culture, the current “gold standard” TB diagnostic, detects only about 30% of clinically diagnosed TB patients in China. AFB smear tests exhibit an extremely high false-negative rate (26), but due to the long turn-

Serum Antigen Detection for Accurate TB Diagnosis



**Fig. 3.** Serum CFP-10 (red) and ESAT-6 (green) concentrations (CFP-10 + ESAT-6) in each clinically confirmed TB case, with patients grouped according to their *Mtb* culture status and disease site. Results indicate an average of 3 sample replicates per subject.



around time required for culture results, AFB smear is still widely used as a rapid initial screen for active TB. Due to this poor clinical sensitivity, suspected TB cases with negative culture and AFB smear results are often subjected to additional tests and/or empirical anti-TB therapy, resulting in additional costs and potentially unnecessary drug burden and toxicity for patients without TB.

In this study, *Mtb* culture and AFB smear results had 48.2% and 8.6% clinical sensitivity, respectively, across all TB cases, but their sensitivity decreased dramatically in extrapulmonary cases for AFB smear (0.0%). NanoDisk-MS clinical sensitivity did not differ for pulmonary vs extrapulmonary TB, nor was it impacted by culture or AFB status, strongly indicating that this blood-based biomarker approach can improve TB diagnosis in this diagnostically challenging patient cohort. Further, intergroup comparison of combined biomarker concentration indicated similar circulating levels regardless of bacterial load and disease site, supporting the utility of our serum biosignature across a broad spectrum of TB manifestations. *Mtb* culture, AFB smear, and radiographic results are qualitative and reflect bacilli present only in a given sample or image. NanoDisk-MS results,

however, quantify circulating *Mtb* antigens as a measure of the systemic level of active *Mtb* bacilli, independent of the disease site(s), and should therefore differ among TB cases who have similar culture, smear, or radiographic findings, but different TB disease severity or extent.

The diagnosis of extrapulmonary and paucibacillary TB has not been greatly improved by the Xpert MTB/RIF assay, an automated molecular assay developed for rapid diagnosis of TB and rifampicin resistance (6–8). Indeed, the WHO has acknowledged the low-quality evidence for the use of Xpert to diagnose extrapulmonary TB, which accounts for about 25% of all TB cases (27). Further, previous publications suggest that Xpert MTB/RIF has low clinical sensitivity in culture-negative TB (28, 29). One study observed markedly reduced clinical sensitivity in culture-negative vs culture-positive pulmonary (25.0% vs 86.2%) and extrapulmonary (29.4% vs 67.7%) TB cases (26), suggesting that culture status has a profound effect on DNA-based diagnosis of both pulmonary and extrapulmonary TB infections. NanoDisk-MS thus appears to dramatically improve on culture and Xpert MTB/RIF both for its ability to utilize patient serum, rather than sputum or invasive biopsy specimens, and for its robust perfor-

## Serum Antigen Detection for Accurate TB Diagnosis

mance, which is unaffected by anatomical site (pulmonary vs extrapulmonary) or bacillary load (*Mtb* culture status).

NanoDisk-MS exhibited 95.8% clinical specificity in 96 non-TB individuals in this study. However, it is not clear why 4 of these patients had detectable *Mtb* antigen levels, as our MS-based method is highly specific for its target peptides. While these results may indicate actual false-positive events, it is also possible they represent sub-clinical infections with TB or specific strains of 2 NTM species (*M. kansasii* and *M. marinum*) with homology to the *Mtb* CFP-10 target peptide. Thus, clinical evaluation is still necessary for a final diagnosis of active TB based on positive NanoDisk-MS results. These 2 NTM should be rare (<0.5% suspected TB cases) in our population (30), but due to the lack of systematic clinical data for the non-TB individuals in this study we cannot provide definitive explanations for the NanoDisk-MS positive results in this group.

NTM infections, which have lower incidence than TB, can cause false-positive TB diagnosis owing to their similar clinical symptoms and bacteriological results. Further, several NTM species, including several of the more clinically common species, can also secrete homologs of CFP-10 and ESAT-6, which might complicate diagnosis. We have previously reported that the CFP-10 and ESAT-6 peptides analyzed by NanoDisk-MS are highly *Mtb*-specific, however, and our results correctly excluded 1 patient with a clinical diagnosis of active TB, who was later determined to have an NTM infection (*M. intracellulare*) by *groEL2* gene sequencing.

The NanoDisk-MS assay was developed to permit the analytically sensitive multiplex quantification of serum CFP-10 and ESAT-6 concentrations in suspected TB cases to allow rapid diagnosis of active TB with high clinical sensitivity and specificity, irrespective of disease manifestation or *Mtb* concentration at the disease site(s). We detected both these *Mtb* virulence factors in our study population, but CFP-10 was observed with markedly higher frequency than ESAT-6. We did not observe any correlation between the CFP-10 and ESAT-6 concentrations within each patient. This might be a result of variable expression of ESAT-6 and CFP-10 in different strains of *Mtb* (31, 32). A recent study has also demonstrated that the host protein  $\beta_2$ -microglobulin can bind ESAT-6 and mask a tryptic cleavage site (33) that is required to generate the ESAT-6 1900.95 *m/z* peptide recognized by our NanoDisk-MS assay (34). The differences in CFP-10 and ESAT-6 detection rates may be due to a combination of these 2 factors.

NanoDisk-MS differs from other current TB diagnostic tests by providing quantitative results. We anticipate that longitudinal NanoDisk-MS results may there-

fore be useful for rapidly evaluating treatment response and predicting clinical outcomes of TB patients, which currently rely on culture conversion or nonstandardized physician evaluations. Serial quantification of serum *Mtb* antigens may also be useful for predicting the likelihood of drug resistance and disease recurrence. We did not observe correlation between CFP-10 and ESAT-6 concentrations, and diagnosis and treatment monitoring thus require separate assessment of these 2 biomarkers. Many hospitals and public health laboratories routinely employ MALDI-TOF MS for microbial identification (35–37), which should ease the translation of this method. However, development and optimization of a long-term storage method for antibody-conjugated NanoDisks is required before this platform is suitable for use in clinical studies. Ongoing development of less expensive, portable MS platforms, designed for use in resource-limited areas, may allow this approach to achieve widespread clinical application in all areas with high TB-burden (38, 39). Overall, our results support the further evaluation of NanoDisk-MS to detect circulating CFP-10 and ESAT-6 concentrations in patients with suspected TB as a means for rapid and accurate TB disease diagnosis in adults and in children who have paucibacillary disease.

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## Appendix B

### Standard operating procedures

#### **B.1 Standard Operating Procedure 1: Gastric aspirate collection in children**

##### **1. BACKGROUND**

Gastric aspirate specimens are collected in young children (typically below 5 years of age) who cannot spontaneously expectorate a sputum specimen for TB microbiology investigations. Gastric aspirates are used to recover mycobacteria in the sputum that may have been swallowed during the night. The gastric contents must be neutralised to prevent destruction of tubercle bacilli by the acidity.

##### **2. OBJECTIVES**

This standard operating procedure (SOP) describes the procedure to collect gastric aspirate specimens.

##### **3. SCOPE**

Studies needing to collect gastric aspirates.

**4. PROCEDURES:****Equipment required for gastric aspirate collection****Table 10.1: List of equipment**

1. Disposable Gloves (non-sterile)
2. Particulate respirator masks (N95 or equivalent)
3. Disposable aprons
4. Disposable linen saver
5. Paper towel
6. 3 bed sheets or surgical drapes: one for the bed, one for wrapping child into and one for putting over the child
7. Cotton wool to clean nose (saline / sterile water is used)
8. Normal saline (0.9% NaCl) or sterile water in single use vials (5 ml better to avoid waste)
9. Oxymetazoline drops (optional)
10. Remicaine (Lidocaine hydrochloride) jelly (optional)
11. Nasogastric tube-NGT (6-10 French): preferred are Ryles (longer) or Levin tubes
12. 5, 10 and 20 cc syringe for gastric aspirate collection
13. 1 cc syringe for bicarbonate titration
14. Stethoscope
15. Sample container. Containers may be study/lab specific. If not, example: Falcon 50 ml conical centrifuge tube skirted (self-standing)
16. pH strips 0 to 14 (pH strips are more precise than Litmus paper) (From AEC-Amersham, Fisherbrand 10642751, or from Sigma-Aldrich; Z111821-1EA; PCode 1001904979) (not required if sample sent to BARC)
17. Plastic racks for 50ml tubes (Manufacturer: B and M Scientific Ref Code MRTR1550 )
18. Sodium Bicarbonate (4%) solution for bedside neutralization provided by DTTC laboratory if sample sent to NHLS-Tygerberg (or other NHLS lab). If sample is sent to BARC, BARC will provide bicarbonate in powder form and instructions for use
19. Laboratory requisition forms
20. Laboratory specimen barcodes
21. Laboratory transport bags

22. Disinfectant for surfaces
-------------------------------

**Table 10.2. Order of and Fasting time between respiratory samples**

Children who cannot expectorate sputum		
Order	Sample type	Minimum hours nil per os (NPO)
1	Gastric aspirate	4 hours
3	Induced sputum	2 hours
Children who can expectorate sputum		
Order	Sample type	Minimum hours NPO
1	Expectorated Sputum early AM <sup>A</sup>	4 hours
2	Expectorated Sputum spot	2 hours <sup>B</sup>
3	Induced sputum	2 hours

A: Can be done midday if fasted for >4 hours

B: If only 1 specimen collected during the day, no fasting necessary. If 2 specimens collected in one day, there should be at least 2 hours in between specimens.

Example: if one morning sputum is taken after 4 hours of fasting, one must wait another 2 hours to collect an expectorated spot sputum (rationale; otherwise it is the same sputum, as everything should have been expectorated- time is needed to “develop” more sputum).

## 5. GASTRIC ASPIRATION COLLECTION

1. The caregiver will be instructed regarding overnight fasting of at least 4 hours before obtaining the early morning gastric aspirate (GA). Children over 1 year of age can usually cope with a full overnight fast (6-8 hours), and this is preferable over a shorter fast (4 hours). The procedure is preferably performed early in the morning when the child comes for a study visit or in the ward if child is an in-patient. The procedure may also be performed during the daytime, as long as the child has been kept nil per os (NPO) for minimum 4 hours. If the child is an outpatient, the caregiver should be contacted the day before the procedure and informed about the fasting period.
2. Conduct this procedure in a sputum/procedure room with adequate ventilation or negative pressure (see CM005 Infection Prevention and Control SOP and Table 3).

**Table 10.3. Time required to clear the air of *M. tuberculosis* after the source patient leaves the area or when aerosol-producing procedures are completed (see reference 1).**

Air changes per hour (ACH) and time required for removal efficiencies of 99% and 99.9% of airborne contaminants*		
ACH	Minutes required for removal efficiency†	
	99%	99.9%
2	138	207
4	69	104
6	46	69
12	23	35
15	18	28
20	14	21
50	6	8
400	<1	1

\* This table can be used to estimate the time necessary to clear the air of airborne *Mycobacterium tuberculosis* after the source patient leaves the area or when aerosol-producing procedures are complete.

† Time in minutes to reduce the airborne concentration by 99% or 99.9%.

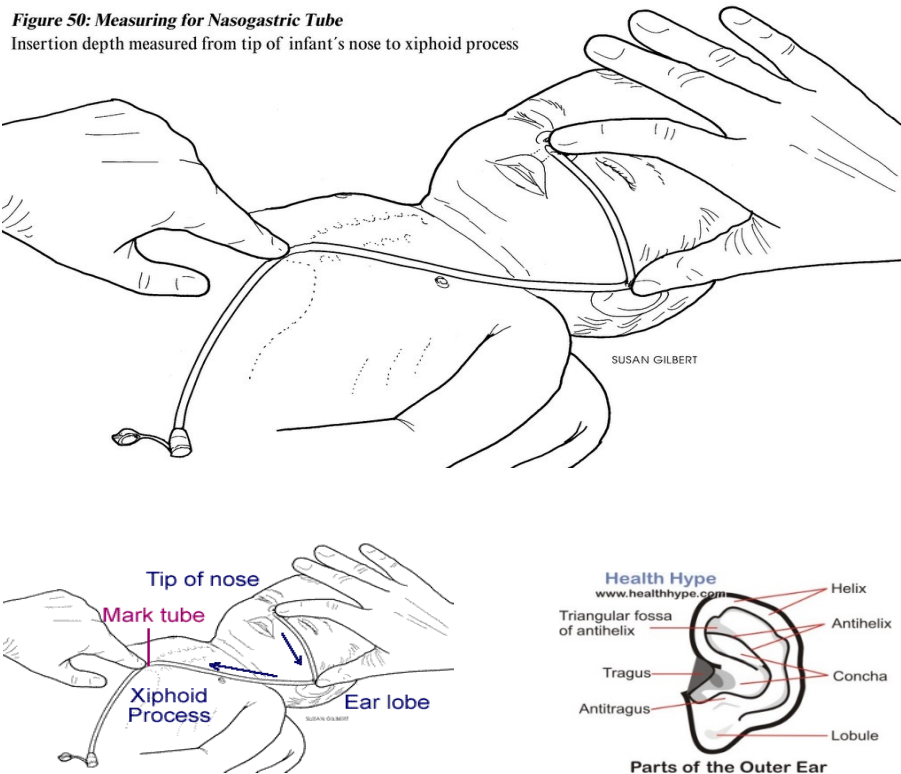
3. Use an assistant to help as this procedure requires 2-3 people.
4. Prepare all equipment for the procedure.
5. Open the specimen container (Falcon tube) and put the container on a rack so that it is vertical.
6. Disinfect all working surfaces including bed with 70% alcohol solution or sodium hypochlorite (bleach) solution at a strength of 10000 parts per million (see Table 3).
7. Place a drape over the bed. Use one drape to immobilize the child and one to cover the child leaving the head exposed.
8. Position the child in decubitus supine position with the help of an assistant.
9. Optional: instil 2 drops of oxymetazoline into each nostril to induce vasoconstriction and prevent epistaxis. Instil carefully without touching the child/nostrils with the dispenser.
10. Measure the distance of the nasogastric tube to the stomach (from nose to the tragus of the ear and to xiphisternum): this estimates the distance that will be required to insert the tube. See Figure 1 below.
11. Open the plastic sleeve of the nasogastric tube. If used, squeeze a blob of remicaine jelly into the inside of the sleeve and coat the outside of the nasogastric tube with the jelly by sliding it along the blob.

12. Placing child's face in the "sniffing air" position, the nasogastric tube is passed from the nose into the stomach.
13. Attach a syringe (10mL if using Levin or 20 ml if using Ryles tubes) to the nasogastric tube (size 6-10 French, depending on the size of the child).
14. Check NGT position by pushing some air from the syringe into the stomach (3-5ml), and listening with a stethoscope over the stomach.
15. Withdraw (aspirate) gastric contents using the syringe attached to the nasogastric tube.
16. Aspirate stomach contents gently and steadily, with the child in each of 3 positions: head central, left lateral and right lateral. Allow a few seconds before aspirating after changing position. If no fluid is aspirated, push tube 1-2cm deeper or pull out 1-2cm shallower, and aspirate.
17. The recommended volumes to collect are higher than sputum: 5 to 10 mL should be collected at a minimum.
18. In the rare event that less than 1ml is aspirated, a gastric lavage can be performed:
  - a. Sterile water (alternative: preservative free normal saline) 10 ml will be inserted into the tube, left for three minutes, and then aspirated until a minimum of 5-10ml aspirate is obtained.
  - b. If no fluid is aspirated, instil additional 10 ml of sterile water and aspirate again. If still unsuccessful, repeat this up to 3 times in total.
19. Transfer the full volume of gastric fluid after each aspiration from syringe into a sterile container (Falcon tube). The same syringe may be used for each aspiration.
20. When no more gastric fluid can be aspirated, gently withdraw the nasogastric tube and discard it into a biosafety container. If necessary, clean the child's face with paper towel and lift the child from the bed. The assistant can hold the child until the nurse has fully completed the procedure, or the child can be returned to the caregiver.
21. If required by study- specific protocols or if sample is sent to NHLS-Tygerberg, measure the initial pH of the fluid. This step is not required when the sample is sent to BARC.
  - a. Put the sterile container (Falcon tube) on the tube rack
  - b. Prepare a pH strip and put it on a paper towel on the linen saver.
  - c. Using the sterile pipette or the tip of a sterile needle, get one drop of the GA from the tube, and put this drop on the pH strip.
22. Interpretation of pH result and neutralisation:
  - a. If the pH is  $\geq 6$ , no need to do anything

- b. If the pH is < 6, draw up at least 2 ml of the 4% bicarbonate solution in a small syringe or pipette and add 0.1 ml at a time, measuring the pH in between additions, until correct pH is reached (pH 6-7). Do not touch the inside of the gastric aspirate container when adding the bicarbonates.
23. Screw the lid on the tube and clean the outside of the tube with alcohol swabs.
24. Label the tube with the appropriate barcode(s) and complete the appropriate lab forms/CRFs.
25. Place in sample bag, seal the bag and keep at 2-8°C until transport to the laboratory. The specimen must be transported to the lab in a cooler box with ice bricks to keep it at 2-8°C during transport.

### Figure 10.1: Measuring for nasogastric tube

Image from <http://nursingfornurse.blogspot.co.za/2005/11/how-to-confirm-nasogastric-tube-ngt.html>



## 6. SOP TRAINING

1. The PI or designee arranges training on SOPs for:

- a. New and existing staff members (all nurses and doctors working on the study should receive training)
  - b. Retraining on revised SOPs
  - c. Retraining of a staff member in the event of a SOP violation/continuous SOP violations
2. There are two mechanisms for SOP training: attendance at a formal SOP training session, and self-study.
  3. Once staff members have read/trained on a SOP, they must sign the signature log which is attached to each SOP as proof of training/self-study.
  4. These signature logs are kept in the study coordinator's office until all relevant staff have signed, after which it is filed together with the Master Copy in the office of the CRS Coordinator.

## 7. REFERENCES

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## 8. DEFINITIONS

None

## 9. REVIEW AND REVISION

### Revisions May 2017:

#### Section 5.1

- Oxymetazoline drops and Remicaine (Lidocaine hydrochloride) jelly specified as optional
- Sentence added regarding the type of bicarbonates used depending on the receiving laboratory (NHLS vs BARC)

#### Section 5.2

- Sentence added regarding the need to verify the pH depending on the study and/or receiving laboratory
- Table with number of air changes per hour added
- Edits in text throughout for clarity

## **B.2 Standard Operating Procedure 2: Sputum induction collection in children**

### **10. BACKGROUND**

Sputum induction is typically used in patients who are unable to produce sputum spontaneously. The patient inhales nebulised hypertonic saline solution, which liquefies airway secretions, promotes coughing and allows expectoration of respiratory secretions. In young children, nasopharyngeal aspiration is usually required for sputum collection.

### **11. OBJECTIVES**

This SOP describes the procedure for sputum induction.

### **12. SCOPE**

DTTC Studies



**13. PROCEDURES:****Equipment required for sputum induction****Table 10.4. List of equipment**

1.	Nebuliser
2.	For each patient (discard after use): Tubing and face mask (Example: HiCare pediatric elongated)
3.	For each patient (discard after use unless used by same child later): Spacer (Example: Cipla Zerostat VT spacer) with mask if child < 5 years (Example: Cipla Baby Mask)
4.	Salbutamol (100µg/puff)
5.	Suction apparatus
6.	For each patient (discard after use): silicone tubing for suction
7.	Pulse oximeter
8.	Hypertonic (5%) (can be 3%) saline solution. Treatment Response team uses 200 ml bag and takes 5 ml with sterile syringe. To store in fridge.
9.	Disposable gloves and N95/P2 respirator masks
10.	Paper towels
11.	Kidney dish
12.	Sputum specimen container (screw-top). Containers may be study specific. If not, example: Falcon 50 ml conical centrifuge tube skirted (self-standing)
13.	Laboratory forms
14.	Laboratory bags
15.	5ml and 10ml syringes
16.	19G needle
17.	Sharps container
18.	Sterile 6/7/8 G mucus extractor or nasogastric catheter

**14. ORDER OF AND FASTING TIME BETWEEN RESPIRATORY SAMPLES:****Table 10.5. Order of and Fasting time between respiratory samples**

Children who cannot expectorate sputum		
Order	Sample type	Minimum hours nil per os (NPO)
1	Gastric aspirate	4 hours
2	NPA	2 hours
3	Induced sputum	2 hours
Children who can expectorate sputum		
Order	Sample type	Minimum hours NPO
1	Expectorated Sputum early AM <sup>A</sup>	4 hours
2	Expectorated Sputum spot	2 hours <sup>B</sup>
3	Induced sputum	2 hours

A: Can be done midday if fasted for >4 hours

B: If only 1 specimen collected during the day, no fasting necessary. If 2 specimens collected in one day, there should be at least 2 hours in between specimens.

Example: if one morning sputum is taken after 4 hours of fasting, one must wait another 2 hours to collect an expectorated spot sputum (rationale; otherwise it is the same sputum, as everything should have been expectorated- time is needed to “develop” more sputum).

**15. CONTRA-INDICATIONS/PRECAUTIONS**

1. As hypertonic saline causes bronchoconstriction, the procedure should only be performed after pre-medication with salbutamol and under medical supervision in patients with asthma or severely impaired lung function.
2. As the procedure induces coughing, it should not be performed in patients in whom severe coughing may be harmful, including patients with:
  - a. Unstable respiratory state: acute respiratory distress, pertussis-syndrome, hypoxia (Oxygen saturation <92% in room air), pneumothorax.
  - b. Unstable cardiovascular status (including untreated cyanotic heart disease)
  - c. Recent surgery: attending surgeon to assess

- d. Any condition where the patient is unable to protect the airway e.g. depressed level of consciousness

## 16. SPUTUM INDUCTION PROCEDURE

17. Sputum induction is performed by a research nurse trained in this technique, and is undertaken after a minimum of 2h fast.
18. Clinical evaluation form is completed before procedure, documenting general observations and chest auscultation. (See number 7 below). Detection of severe respiratory distress or severe tachycardia is a contra-indication for the procedure.
19. Oxygen saturation and pulse rate must be monitored throughout the procedure. Stop the procedure in event of a fall in saturation <90% and a pulse rate >180 or <100 bpm
20. Child is pre-treated with 200µg salbutamol via metered dose inhaler with attached spacer to prevent bronchoconstriction. This is done by placing the assembled metered dose inhaler/spacer/mask onto child's mouth and nose. Child is allowed to settle until breathing freely. One puff is activated, keeping the mask in the same position, and the child is allowed to breathe 4-5 times. Mask is removed.
21. The child's nose is cleaned with saline and cotton wool to remove nasal mucus prior to sputum induction. If nasal secretions are thick, a soft catheter size F6/7 is used for suctioning and is discarded immediately afterwards.
22. A jet nebuliser attached to oxygen at a flow rate of 5-7 L per minute delivers 5 mL of 5% sterile saline for 15 minutes or until child starts to cough.
23. In young children who cannot expectorate spontaneously:
  - a. Once the child starts to cough, sputum is obtained by suctioning through the nasopharynx with a sterile mucus extractor of catheter size 6 or 7. Pass the catheter up to a length equal to the distance from nostril to tragus of the ear, without applying suction. Once in position, apply suction. Catheter can be moved a little and turned to facilitate cough reflex and aspiration of sputum. Suction pressures by ages, according to the American Association for Respiratory Care (AARC) Guidelines for appropriate sub-atmospheric nasotracheal suctioning pressures:
    - Neonates: 60 - 80 mm Hg (0.079- 0.10 Bar)
    - Infants: 80 - 100 mm Hg (0.079- 0.13 Bar)
    - Children: 100 - 120 mm Hg (0.13- 0.15 Bar)

- Adults: 100 - 150 mm Hg (0.13- 0.19 Bar)

Negative pressures should not exceed 150 mm Hg as higher pressures have been shown to cause trauma, hypoxemia and atelectasis. Once sputum is obtained, the catheter is withdrawn from the nose. DO NOT aspirate as the catheter passes through the nose. If mucus is to be obtained from the oral cavity as well, the mouth should be rinsed/wiped with anti-bacterial, non-alcohol-based mouth wash prior to the entire procedure, in order to avoid contamination.

- b. If the child does not cough after nebulisation, chest percussion is done over the anterior and posterior chest wall. Mucus is then extracted as (7) above. Nebulisation and chest percussion followed by nasopharyngeal aspiration can be repeated if an inadequate sample is obtained (volume <1mL).
24. In older children who can expectorate spontaneously:
    - a. Once the child starts to cough, encourage the expectoration of sputum into a sputum container. The child should continue to expectorate until no more sputum can be produced. Nebulisation can be repeated if an inadequate sample is obtained (volume <1mL or watery sample indicative of saliva)
    - b. If the child does not cough after nebulisation, encourage the child to perform deep breathing. The child can also be made to jump or run on the spot if clinically stable and able to do so. Chest percussion is done over the anterior and posterior chest wall. Encourage the child to expectorate as in 8a above.
  25. Sputum induction is best done approximately 6 h after the early morning gastric lavage.
  26. Spacers and nebuliser equipment will be cleaned after every use:
    - a. Clean the nebulising machine and other surfaces with 70% alcohol after each use
    - b. Discard disposable material (masks, tubing, spacers)
    - c. Do not re-use spacers and masks unless for same patient
  27. Complete clinical evaluation form after procedure. Any new signs of respiratory distress not settling after 5-10 minutes of supplemental oxygen via face mask must be reported immediately to attending clinician. SAE form must then be completed.
  28. Following completion of the procedure:

- a. Wipe the specimen jar with alcohol/chlorhexidine to prevent cross-infection and ensure that the specimen jar is labelled. Make sure to keep the sample at 4-8°C until transport.
- b. Complete the appropriate forms as described by the study specific SOP on Handling and Transporting Samples
- c. Offer the child a snack after the procedure

## 29. SOP TRAINING

1. The PI or designee arranges training on SOPs for:
  - a. New and existing staff members
  - b. Retraining on revised SOPs
  - c. Retraining of a staff member in the event of a SOP violation/continuous SOP violations
2. There are two mechanisms for SOP training: attendance at a formal SOP training session, and self-study.
3. Once staff members have read/trained on a SOP, they must sign the signature log which is attached to each SOP as proof of training/self-study.
4. These signature logs are kept in the study coordinator's office until all relevant staff have signed, after which it is filed together with the Master Copy in the office of the CRS Coordinator.

## 30. REFERENCES

1. Carroll NM, Richardson M, Engelke E, de Kock M, Lombard C, van Helden PD. Reduction of the rate of false-positive cultures of *Mycobacterium tuberculosis* in a laboratory with a high culture positivity rate. *Clin Chem Lab Med* 2002;40(9):888-92.
2. Talbot EA, Williams DL, Frothingham R. 1997. PCR identification of *Mycobacterium bovis* BCG. *J Clin Microbiol*; 35:566-9.
3. Warren RM, Streicher EM, Sampson SL, van der Spuy GD, Richardson M, Nguyen D, Behr MA, Victor TC and van Helden PD. 2002. Microevolution of the direct repeat region of *Mycobacterium tuberculosis*: implications for interpretation of spoligotyping data. *J Clin Microbiol.* 40: 4457-4465.
4. Zar H, Hanslo D, Apolles P, Swingler G, Hussey G. Induced sputum versus gastric lavage for microbiological confirmation of pulmonary tuberculosis in infants and young children: a prospective study. *Lancet* 2005: 365:130-134.

**31. DEFINITIONS**

None

**32. REVIEW AND REVISION**

This SOP is reviewed biennially or as required by the PI or designee.

## **B.3 Standard Operating Procedure 3: Nasopharyngeal aspiration in children**

**33. BACKGROUND****34. TUBERCULOSIS DETECTION**

To diagnose pulmonary tuberculosis (PTB) in children, it remains very important to seek bacteriological confirmation. The rate of bacteriological confirmation can be increased by collecting different types of specimens from a child with suspected PTB. A nasopharyngeal aspiration (NPA) is one of the options to collect sputum in young children.

**35. BACTERIAL AND VIRAL DETECTION**

Bacteria, fungi, and viruses can all cause respiratory tract infections. A NPA is taken to investigate what type of organism is causing respiratory symptoms.

**36. OBJECTIVES**

To collect an NPA for mycobacterial, bacterial and viral studies.

**37. SCOPE**

DTTC paediatric studies

**38. PROCEDURES:****39. ORDER OF AND FASTING TIME BETWEEN RESPIRATORY SAMPLES:**

**Table 10.6. Order of and Fasting time between respiratory samples**

Children who cannot expectorate sputum		
Order	Sample type	Minimum hours nil per os (NPO)
1	Gastric aspirate	4 hours
2	NPA	2 hours

3	Induced sputum	2 hours
Children who can expectorate sputum		
Order	Sample type	Minimum hours NPO
1	Expectorated Sputum early AM <sup>A</sup>	4 hours
2	Expectorated Sputum spot	2 hours <sup>B</sup>
3	Induced sputum	2 hours

A: Can be done midday if fasted for >4 hours

B: If only 1 specimen collected during the day, no fasting necessary. If 2 specimens collected in one day, there should be at least 2 hours in between specimens.

Example: if one morning sputum is taken after 4 hours of fasting, one must wait another 2 hours to collect an expectorated spot sputum (rationale; otherwise it is the same sputum, as everything should have been expectorated- time is needed to “develop” more sputum).

#### 40. EQUIPMENT REQUIRED

1. Suction apparatus
2. Disposable gloves and P2 respirator masks
3. Paper towels
4. Normal saline (0.9%)
5. Oxymetazoline (optional)
6. Sterile CH/ Fr 6/7/8 mucus extractor or nasogastric catheter
7. Cotton wool
8. Kidney dish
9. Laboratory and study forms
10. Laboratory bags

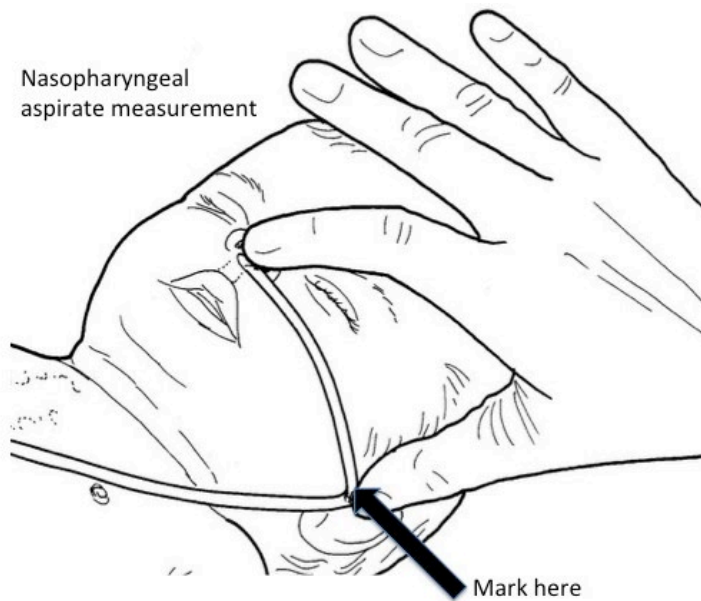
#### 41. PROCEDURE

If other respiratory samples are being collected from the same participant on the same day, then this procedure for NPA collection in young children will be conducted before sputum induction and after gastric aspirate or expectorated sputum collection.

1. The child’s nose is cleaned with saline drops and cotton wool. If old enough, the child can be asked to blow the nose into a tissue. If the nasal mucus is too thick to be removed with the measures above, it can be suctioned prior to nasopharyngeal

aspiration. A soft catheter size Fr 6/7 is used for suctioning and is discarded immediately afterwards.

2. One drop of oxymetazoline may be instilled into each nostril to prevent nose-bleeds.
3. Two drops of sterile saline are instilled into each nostril.
4. The length of the cannula used for aspirating the NPA sample is measured as the distance from nostril to tragus of the ear (see picture below); then the posterior nasopharynx is suctioned using a soft plastic cannula connected to a mucus trap.



**Figure 10.2. Measuring the cannula for aspirating the NPA sample**

Suctioning is activated only when the tip of the cannula is in the posterior nasopharynx. When the cannula is passed through the nostrils (during introduction and extraction), the suction is de-activated.

Suction pressures by ages, according to the American Association for Respiratory Care (AARC) Guidelines for appropriate sub-atmospheric nasotracheal suctioning pressures:

- Neonates: 60 - 80 mm Hg (0.079- 0.10 Bar)
- Infants: 80 - 100 mm Hg (0.079- 0.13 Bar)
- Children: 100 - 120 mm Hg (0.13- 0.15 Bar)



- Adults: 100 - 150 mm Hg (0.13- 0.19 Bar)
5. At least 1ml of specimen should be collected.

#### **42. FOLLOWING COMPLETION OF THE PROCEDURES**

1. Wipe the specimen jar with 70% alcohol/chlorhexidine to prevent cross-infection and ensure that the specimen jar is labelled.
2. Complete the appropriate lab forms

#### **43. TRANSPORT OF SPECIMENS TO THE LABORATORY**

1. Specimens will be stored and transported out of direct sunlight, in the fridge and store at 4-8°C until transport.
2. All specimens will be transported to the medical microbiology laboratory and depending on requested test forwarded to the correct department
3. Transport the specimen (in a cool box containing an ice brick) to the lab for processing as soon as possible (at least within 4 hours)

#### **44. REFERENCES**

None.

#### **45. DEFINITIONS**

None.

#### **46. REVIEW AND REVISION**

This SOP will be reviewed biennially or as required by sponsor, the PI or designee.

## **B.4 Standard Operating Procedure 4: Stool collection in children**

#### **47. BACKGROUND**

In the context of TB diagnosis, stool can be collected for acid fast bacilli (AFB) smear, culture, GeneXpert and other nucleic acid amplification tests.

Stool collection methods for bacteriology and parasitology testing require that stool should not be collected/mixed with:

1. mineral oil, barium, bismuth, magnesium compounds, mineral oil, petroleum jelly (Vaseline), etc. Glycerin suppositories are usually accepted.
2. water/toilet water
3. urine

(Adapted from: <http://www.cdc.gov/dpdx/diagnosticProcedures/stool/specimencoll.html>)

It is possible that these compounds may also render the stool specimens unsatisfactory for examination of TB using molecular methods but very little data is available. In doubt, it is best to collect stool specimens using the same recommendations to maximise the yield.

For young children in diapers, it is recommended to line the inside of the diaper with plastic wrap/cling film or a waterproof to keep the stool sample from being absorbed into the diaper. It is also important to avoid having urine mixed with the stools. Various collection methods are described below.

#### 48. **OBJECTIVES**

This SOP describes the procedure to collect stool specimens in the context of TB diagnosis.

#### 49. **SCOPE**

DTTC Paediatric Studies

#### 50. **PROCEDURES:**

##### 51. **INFECTION PREVENTION AND CONTROL PRACTICES**

Stool specimen collection is generally regarded as a low risk procedure in terms of potential transmission of TB; however, the patient/parent/caregiver infectiousness status can be uncertain at the time of specimen collection. It is therefore best to proceed in an area with adequate ventilation and using adequate respiratory protection.

**52. EQUIPMENT REQUIRED****Table 10.7. List of equipment**

1. Disposable Gloves (non-sterile)
2. Particulate respirator masks (N95 or equivalent)
3. Disposable aprons
4. Disposable linen saver
5. Sample container. Containers may be study/lab specific. If not, example: Fecal Cup With Spoon (25 ml) (LASEC product number: PLPS109148)
6. Urine bag (if used): Containers may be study/lab specific. If not, example: Order from Mo-Med Distributors (ph: 021-447-9393).No product number, "Urine collectors 200 ml". Other option: Order from Akacia Medical Item Code: PC100110. Nappi Code : 426960002. Supplier Code : GCU240101
7. Waterproof (currently obtained from PEP store: unbranded with no manufacturer / distributor details, stored loose in the store in a box, @<R2.00 per waterproof)
8. Labels and indelible marker pen
9. Leak-proof biohazard bag and absorbent material for transport
10. Transport cooler box
11. Specimen transfer and requisition forms
12. Potty
13. Cling-wrap (transparent food film)
14. Opaque carrier bag to protect dignity

**53. COLLECTION TECHNIQUES FOR CHILDREN IN DIAPERS****54. USING WATERPROOF SHEETING/CLING FILM/PLASTIC WRAP OVER DIAPER**

1. Cover the diaper's interior with waterproof sheeting/cling wrap to prevent stool from soaking (see Figure 3).
2. Collect stool as described below (COLLECTION OF STOOL)



**Figure 10.3. Cling wrap/plastic film over diaper (Image from reference 2)**

#### **55. USING URINE BAG TO COLLECT URINE**

1. Remove the child's diaper.
2. Put a urine bag over the perineum to collect urine and therefore avoid urine contaminating the stool sample.
3. Replace the diaper with waterproof diaper cover/underpants (typically used over the conventional diaper to prevent leakage of stool/urine). You may put a normal diaper OVER the waterproof one to stop any urine or stool from spilling out. As soon as stool is passed, take both diapers off carefully.
4. Collect stool as described below (COLLECTION OF STOOL)

#### **56. USING URINE BAG TO COLLECT STOOL**

1. This technique is optimal if the child has loose or watery stool
2. Remove the child's diaper.
3. Place a urine collection bag over the baby's anus.
4. Put a normal diaper
5. Collect stool as described below (COLLECTION OF STOOL)

#### **57. COLLECTION TECHNIQUES FOR OLDER CHILDREN:**

##### **USING CLING FILM/PLASTIC WRAP OVER TOILET**

1. This technique should be used in children who are already toilet-trained
2. Place a clean cling film/plastic wrap over the rim of the toilet (see Figure 2)
3. Collect stool as described below (COLLECTION OF STOOL)



**Figure 10.4. Cling film/plastic wrap over toilet (from reference 2)**

#### **58. USING POTTY/BED PAN**

1. Let the child first pass urine into the toilet. Then allow the child to pass stool into a potty/empty plastic container/bed pan.
2. Collect stool as described below (COLLECTION OF STOOL)

#### **59. COLLECTION OF STOOL**

1. Use a spoon or spatula to transfer the stool sample to the container. If using wooden applicator stick to transfer stool, do not leave the wooden stick in the container as it may absorb moisture.
2. Make sure no urine, water, soil or other material gets in the container. Attempt should be made to collect a specimen that consists primarily of stool when only a mixture of stool and urine is available.
3. Ensure that the specimen containers are sealed well.

#### **60. COLLECTION OF STOOL SAMPLES FROM THE CHILD'S HOME**

1. In some instances it may be necessary for a caregiver to obtain a stool sample from the child at home, which would then need to be collected by the study team.
2. Clear and simple verbal instructions regarding the method of stool collection (as outlined above) should be given to the caregiver by the study nurse or counsellor.
3. Make sure the caregiver has the correct number of specimen containers to take home.
4. The caregiver or an older child may wish to take protective wear with them to make the process more acceptable. This may include:
  - a. Respirator mask
  - b. Disposable gloves
  - c. Opaque carrier bag (to be used to transport containers back to the research site and to protect the participant's dignity).

5. Encourage the caregiver to put the stool sample in the collection sample containers provided by the study team, and to note the time of stool collection.
6. When the driver collects the stool sample, he / she should
  - a. Wearing gloves, ensure that the collection sample container is sealed and disinfect the outside with 70% alcohol.
  - b. Note the time of stool collection
  - c. Label the collection sample container with the participant's study specific number using a permanent marker

#### **61. PREPARATION OF SPECIMEN AND TRANSPORT TO THE LABORATORY**

1. Label the tube and complete forms as described in the study specific SOP on Handling and Transporting Samples.
2. Place in sample bag, seal and keep at 2-8°C until transport to the laboratory. The specimen must be transported to the lab as soon as possible in a cooler box to keep it at 2-8°C during transport.
3. Respect the maximum accepted time between specimen collection and arrival in the laboratory: refer to the study specific SOP on Handling and Transporting Samples

#### **62. REFERENCES**

1. CDC: <http://www.cdc.gov/dpdx/diagnosticProcedures/stool/specimencoll.html>
2. Seattle Children's Hospital: [www.seattlechildrens.org/pdf/PE810.pdf](http://www.seattlechildrens.org/pdf/PE810.pdf)

#### **63. REVIEW AND REVISION**

This SOP is reviewed biennially or as required by the PI or designee.

#### **64. APPENDICES**

None

## **B.5 Standard Operating Procedure 5: Phase 2A standard**

### **operating procedures Xpert Paediatric stool study**

#### **65. AIM**

The aim of this phase is to assess the performance of three selected volumes (0.6g, 1.2g and swab) for the Xpert stool processing method using the sample preparation buffer selected on phase 1b (SBP2).

#### **66. PROCEDURES:**

**67. EQUIPMENT**

1. GeneXpert System
2. Min. 0.1g-5g weighing scale
3. Vortex Mixer

**MATERIALS**

1. Xpert MTB kits (cartridges, sample reagent and sterile disposable transfer pipettes)
2. MTB Rif sensitive working solution (EQA material, supplied by WHC/NHLS)
3. Stool processing filter unit (supplied by Priya)
4. Sample Processing Buffer 2 (SPB2, supplied by Priya)
5. Glass beads (Supplied by Priya)
6. Sterile flocked swabs (LH-4-30)
7. Sterile Falcon tube and test tube rack
8. Additional transfer pipettes
9. Timer
10. Laboratory Worksheet/CRF form
11. Labels and permanent marker
12. Miscellaneous (Disposable gloves, cleaning solution, etc.)

**PRECAUTIONS, USE AND STORAGE**

Safety Precautions: Universal precautions should be followed when handling all biological specimens. Wear appropriate personal protective equipment and wash hands thoroughly after handling specimens and reagents. When in doubt, refer to the safety procedures set forth by your institution for working with chemical and biological samples. Follow your institution's guidelines for proper disposal of used cartridges and specimens.

Specimen Use and Storage: Specimens utilized during this phase will be collected from children with suspicion of TB and will be either processed immediately after collection or stored at refrigerated conditions. A total of up to 100x2 samples collected at site 1 (WHC) and 50x2 samples collected at site 2 (SUN-DTTC) will be processed in triplicates (0.6g vs. 1.2g vs. swab). Each sample will undergo a total of 3 Xpert tests according to the following table:

**Table 10.8. Stool processing protocol**

<b>Protocol – stool preparation</b>			
	<b>Tube 1 – 0.6g</b>	<b>Tube 2 – 1.2g</b>	<b>Tube 3 – swab</b>
Site 1 (WHC)	Up to 100 x 2		
Site 2 (SUN-DTTC)	Up to 50 x 2		
Total	150-300 samples		

Equipment and Material Use and Storage: Store the Xpert MTB/RIF cartridges and reagents at 2-28°C. Do not use reagents or cartridges that have passed the expiration date. Do not open a cartridge until you are ready to perform testing. Use the cartridge within 30 minutes of opening the lid. The cartridge is stable up to 7 days after opening the package. Follow the manufacturer's instructions and standard operating procedures in your laboratory for usage and storage of other routine equipment. Once completed, dispose used equipment into proper biohazard containers. Sample processing buffer should be used within 3 months of preparation.

**TEST PROCEDURES:****SAMPLE PREPARATION (PERFORM IN BSC)**

Please follow the instructions below and refer to the workflow on page 3 of this document.

Tube 1 – 0.6g

1. Weigh 0.6g of stool
2. Add 2 ml of SPB2 buffer
3. Add 2 ml of SR
4. Add approx. 10 glass beads and snap-vortex (5 sec → observe, if needed vortex again for 5 sec → end) to breakdown any clumps or dense material
5. Incubate the sample mixture at room temperature (20-25°C) for 30 minutes
6. Add stool suspension into the stool processing filter unit and place into a sterile Falcon tube
7. Use the attached plunger to squeeze out the liquid

Tube 2 – 1.2g

1. Weigh 1.2g of stool
2. Add 2 ml of SPB2 buffer
3. Follow steps 3 to 7 above (Tube 1)

Tube 3 – swab (after Tube 1 and Tube 2 have been prepared)

1. Place a swab in the stool container, twirl 1-2x and leave in the stool for 20 sec.
2. Transfer the swab to the tube containing 1 ml of SBP2 buffer



3. Snap vortex (5 sec → observe, if needed vortex again for 5 sec → end) to mix and breakdown any clumps/dense material
4. Add 2ml of SR to the sample mixture and shake 15-20x
5. Incubate the sample mixture at room temperature (20-25°C) for 15 minutes

#### **CARTRIDGE PREPARATION AND TEST INITIATION:**

Note: Start testing within 30 minutes of sample addition to the Xpert MTB/RIF cartridge

1. Using a sterile transfer pipette, aspirate 2ml of filtrate/sample mixture (min. 1.8ml). If there is insufficient volume, do not process the sample further.
2. Open the lid of one Xpert MTB/RIF cartridge, and transfer the stool suspension/sample mixture into the open port of the cartridge. Dispense slowly to avoid aerosol formation.
3. Close the cartridge lid, making sure it snaps firmly into place.
4. Briefly, scan the barcode cartridge, and then scan/type the sample ID.
5. Once instructed, load the cartridge into the GeneXpert system module, and start test.

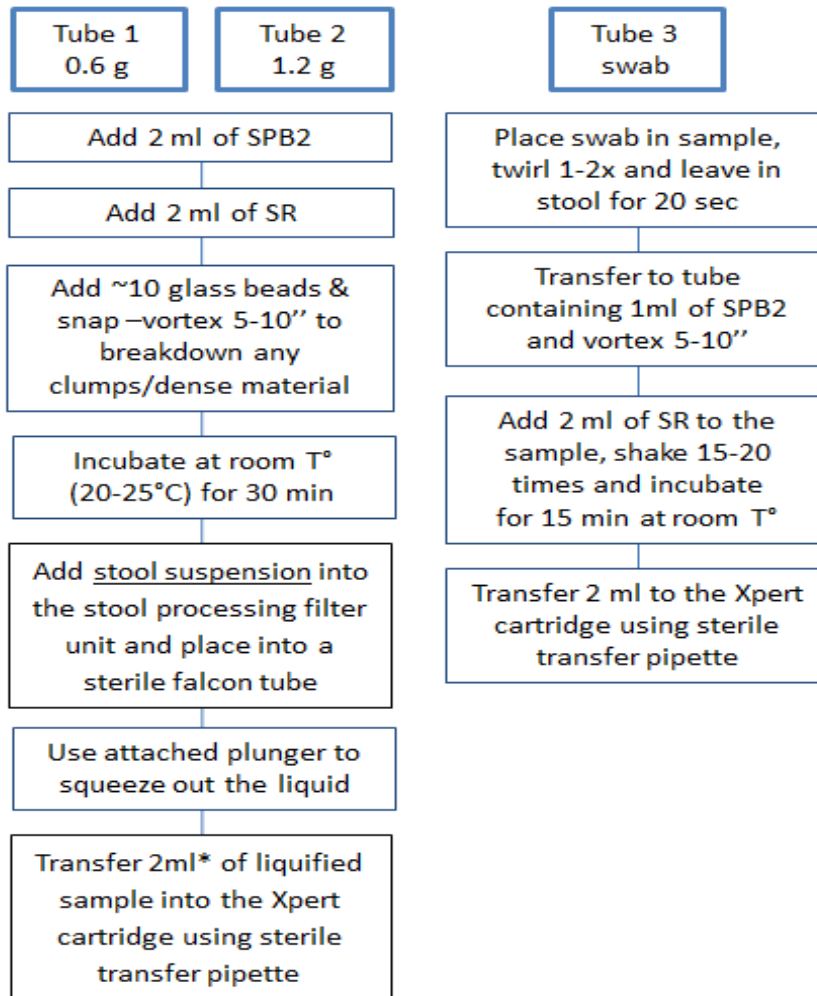
#### **INTERPRETATION OF RESULTS**

1. Once testing is complete, results will be indicated as follows:
  - a) MTB positive or negative
  - b) Semi-quantitation for MTB detection (if MTB positive)
  - c) RIF resistance information
2. If results indicate ERROR, INVALID TEST or NO RESULT, repeat the test if sufficient volume remains using a new Xpert MTB/RIF cartridge.
3. For RIF resistance detected cases, observe the results curves. If the result is due to a probe delay (either probe B or D), the test should be ideally repeated if sufficient volume remains using a new Xpert MTB/RIF cartridge.

#### **RESULTS RECORDING**

Use the Phase 2a CRF to record the results. For each set of samples, indicate the date of sample collection, appearance, and enter the Xpert results accordingly. If the Xpert result was indeterminate (invalid/error/no result) or if Xpert RIF resistance was detected and due to either probe B or D delay, indicate if the test was repeated and if so enter the repeat results. Add any comments at the end.

**SAMPLE FLOW**



\*at least 1.8ml  
 SPB: Stool processing buffer  
 SR: Sample reagent (Cepheid, Xpert MTB/RIF)

**Figure 10.5. Stool processing overview**

## **B.6 Standards Operating Procedure 6: Venous access and blood sample collection in children**

### **68. BACKGROUND**

The responsible study doctor, study coordinator and study nurse should ensure safe and effective venous blood sample collection from participants and ensure that adequate blood volumes are obtained to meet protocol requirements. Patient safety is always a key consideration and blood volumes should also be carefully considered.

### **69. OBJECTIVES**

To describe the procedures of venous access and blood collection on participants in paediatric studies requiring blood sampling and to describe the procedure to obtain adequate blood volumes for non-pharmacokinetic study sampling.

### **70. SCOPE**

This SOP applies to all study staff as indicated on the front page of this SOP.

71.

### **72. EQUIPMENT**

The following equipment should be available for the procedure:

1. Emla 5% anesthetic cream and Tegaderm transparent dressing or equivalents
2. Winged steel needle – 23 or 23 gauge – with an extension tube (butterfly)
3. 2ml and 5 ml sterile syringes or other as required per protocol
4. Latex gloves
5. Webcol prep swabs, Gauze, Plaster, strapping
6. Blood collection tubes
7. Study PID stickers (barcoded if available) and appropriate lab request forms
8. Sample transport bags
9. Sharps bin

### **PROCEDURES**

Make sure to draw blood into the relevant collection tubes in the protocol-specified order, if relevant, to avoid cross-contamination of additives between tubes.

1. Assemble the necessary equipment. Use a butterfly with either a syringe or a vacutainer tube with adapter attached. If using a syringe choose one with a barrel volume of 1-5 ml so as not to collapse the vein.
2. Perform hand hygiene prior to patient contact
3. Select the site for blood draw, preferably a large peripheral vein such as the brachial vein.
4. Palpate the area, locate a vein of a good size that is visible, straight and clear. The vein should be visible without applying the tourniquet. If the other peripheral veins are unsuitable, an experienced sister or doctor may consider using the external jugular vein if there are no contra-indications.
5. Apply topical anesthesia to the skin using Emla 5% or equivalent anesthetic cream, allowing 20-30 minutes for the anesthesia to take effect prior to inserting the needle.
6. Immobilize the participant carefully and in an age-appropriate way, keeping the area selected for venipuncture as still and secure as possible
7. Steps to improve venipuncture:
  - a. Ask a helper to rhythmically tighten and release the child wrist to ensure there is an adequate flow of blood (if arm is used)
  - b. Keep the child warm, which may increase the rate of blood flow.
8. Apply the tourniquet about two finger widths above the venipuncture site.
9. Put on gloves.
10. Disinfect the site using Webcol prep swabs. Do not touch the site once it has been disinfected.
11. If using an evacuated (vacutainer) tube attached to the syringe, attach the end of a winged infusion set to the end of the vacuum tube. Insert the collection tube into the holder until the tube reaches the needle. This is the preferred method to use as it minimizes the risk of a sharp injury occurring. If using a syringe and butterfly, attach the end of the tubing of the butterfly to a syringe of suitable size. NB this method should preferably be used when the vacutainer volume is too large and causes the vein to collapse.
12. Remove the plastic sleeve from the end of the butterfly.
13. Use a thumb to draw the skin tight about two finger widths below the venipuncture site. Do not touch the cleaned site and in particular, do not place a finger over the vein to guide the needle.

14. Enter the vein swiftly at a 30 degree angle. Puncturing the skin 3-5mm distal to (i.e. away from) the vein to allow good access without pushing the vein away.
15. If the needle enters alongside the vein rather than into it, withdraw the needle slightly without removing it completely and angle it into the vessel.
16. If using an evacuated tube, push the collection tube completely onto the needle and allow enough time for the tube to fill, usually until the blood has stopped flowing into the tube, before disconnecting the tube from the holder.
17. If using a syringe attached to the butterfly, gently draw back on the syringe, taking care not to collapse the vein, draw blood slowly and steadily until a sufficient volume for filling the tube is reached. Stabilize the tubes to be filled in the suitable container to minimize the risk of a sharp injury. Now fill the tubes by piercing the protective layer at the top of the tubes with the butterfly needle and waiting for the tubes to fill completely, until the blood stops flowing. Then withdraw the needle very carefully and proceed to fill the subsequent tubes. Take care to stabilize the needle and tube at all times.
18. Remember to invert the tube gently to allow mixing of the blood as applicable to the type of sample collected. Store filled tubes upright at room temperature unless specified otherwise (may need to be stored on ice or frozen samples that are photosensitive may need to have wrapping to protect them from light)
19. It is preferable not to open the caps of tubes and to use the needle to pierce the protective layer of the top of the tube when filling the tubes, so as not to break the vacuum or to compromise the content of the tube.
20. If filling multiple tubes, carefully remove the full tube and replace with another tube taking care not to move the needle in the vein.
21. Once sufficient blood has been collected release the tourniquet then withdraw the needle gently, give the patient a clean gauze or dry cotton-wool ball to press gently on the site. Ask the patient not to bend the arm.
22. Remove the butterfly from the vacuum tube holder. Discard the used needle and syringe or blood-sampling device immediately into the sharps container. This should be clearly visible and within arm's reach.
23. Place necessary items in medical waste bins, including gloves, and then perform hand hygiene.

24. Make sure you store the samples and complete the paperwork as set out in the study specific SOP on Handling and Transporting Samples.

### **73. SOP TRAINING**

1. The PI or designee arranges training on SOPs for:
  - a. New and existing staff members
  - b. Retraining on revised SOPs
  - c. Retraining of a staff member in the event of a SOP violation/continuous SOP violations
2. There are two mechanisms for SOP training: attendance at a formal SOP training session, and self-study.
3. Once staff members have read/trained on a SOP, they must sign the signature log which is attached to each SOP as proof of training/self-study.
4. These signature logs are kept in the study coordinator's office until all relevant staff have signed, after which it is filed together with the Master Copy in the office of the CRS Coordinator.

### **74. REFERENCES**

1. WHO Guidelines on drawing blood: best practices in phlebotomy, dated 2010
2. Health Research Ethics Committee: Guideline for paediatric blood volume for research purposes (Version 1.0 dated 30 November 2015)

### **ABBREVIATIONS**

DTTC Desmond Tutu TB Centre

PK Pharmacokinetics

### **75. DEFINITIONS**

Indwelling: Placed or implanted within the body

Patency: A state of being unblocked

### **76. REVIEW AND REVISIONS**

This SOP is reviewed every two years (biennially) or as required by the Sponsor, Principal Investigator or designee.

**77. APPENDICES**

None

**B.7 Standard Operating Procedure 7: Completing and reading the Mantoux Tuberculin skin test****78. BACKGROUND**

Tuberculosis (TB) is a leading cause of mortality and morbidity in children in the developing world. The World Health Organization (WHO) estimated that in 2013 at least half a million children under 15 years of age developed TB and 80 000 HIV-negative children that died of TB<sup>1</sup>. South Africa has an extremely high burden of TB with an incidence of 860 per 100 000 population in 2013<sup>1</sup>. Children in close contact with an infectious TB source case have a substantial risk of *Mycobacterium tuberculosis* (M.tb) infection and subsequent TB disease in the absence of preventive therapy, especially in the first year after exposure<sup>2</sup>. There is no gold standard for detecting M.tb infection; routinely used tests include the tuberculin skin test (TST) and T-cell based interferon-gamma release assays (IGRAs). Despite known limitations, the TST remains the most commonly used test in high-burden TB settings<sup>3</sup>. The TST has the advantage of being very easy to administer and being inexpensive. The disadvantage is that the test can have false negative and false positive results<sup>3</sup>.

**79. OBJECTIVES**

To test for TB infection.

**80. SCOPE**

This SOP is relevant for all study staff as indicated in the table above

**81. PRODUCT INFORMATION**

Tuberculin diluted 2 TU/ 0.1 mL From Span Diagnostics.

Store at 2-8 °C in the original package in order to protect from light.

**82. PROCEDURES:**

Explain procedure to parent/legal guardian. Explain why the test is given, what is involved in the procedure, when the test should be read and what the possible outcomes can be.

### 83. EQUIPMENT

1. Vial of Tuberculin
2. Single-dose sterile disposable 1mL syringe fitted with a short bevel needle (25 or 26g)
3. Alcohol swabs
4. Cotton wool
5. Sharps disposable container
6. Pen
7. Non-sterile gloves

### 84. PREPARATION

1. Check the vial of Tuberculin for expiry date of the vial.
  - a. The label should indicate the expiration date. If it has been open more than 30 days or the expiration date has passed the vial should be thrown away and a new vial should be used<sup>4</sup>
  - b. If vial closed; to write down date and time of first opening.
2. Wash hands and put on non-sterile gloves.
3. Locate and clean injection site.
  - a. Administer into the left arm unless not possible due to drip/ cast or other problems such as skin disorders.
  - b. Palm-side-up (volar) surface of the forearm, about 5-10cm from the elbow.
  - c. Check if no scars, rashes or bruises in the area that might influence reading at a later stage (then use other arm).
  - d. Clean site using an alcohol swab and allow the site to dry.
4. Prepare the syringe.
  - a. Wipe the top of the vial with a new alcohol swab before drawing up the tuberculin solution.
  - b. Draw the tuberculin solution into the syringe avoiding air entering the syringe. Draw up just over 0.1mL.



- c. Hold the syringe in an upright position, then draw back slightly on the plunger. Tap the syringe lightly to break up air bubbles, then push forward.
    - d. Expel all air and excess fluid from the syringe and needle, leaving exactly one tenth of a milliliter (0.1ml) of tuberculin solution in the syringe.
5. Injection
  - a. Stretch the selected area with thumb and forefinger.
  - b. Inject at an angle of 5-15 degrees (very flat) as it is an intra-dermal injection.
  - c. The entire bevel of the needle should be under and visible the skin (advance  $\pm 3\text{mm}$ ).
  - d. Slowly inject tuberculin solution, while seeing a pale wheal appear.
6. Check the weal
  - a. Measure weal as it should be at least 6mm in diameter (see Figure).
  - b. Circle the weal with a pen.
  - c. The wheal will typically disappear in 10-15 minutes. The size of the wheal is not completely reliable, but if a lot of liquid runs out at the time of injection and there is no wheal, then repeat the injection on the opposite forearm, or on the same forearm as before, but at least 4 cm from the previous injection site.
  - d. It is not unusual for a drop of blood to appear. Lightly blot the blood away with cotton wool, but do not leave it on the injection site as this might interfere with the test.
  - e. Avoid subcutaneous or intramuscular injection of tuberculin. If this occurs, a papule will not develop and the tuberculin skin test should be repeated on the other arm or if the same arm is used the injection site should be separated at least 4 cm from the first injection site.
7. Wash hands, return vial to fridge.
8. Record keeping
  - a. Write in patient file/ road to health card (RTHC) and study file the date/ time of administration, person who administered, site of administration, and type of tuberculin used.
9. Reading of TST

This should be done 48-72 hours after administration.

  - a. Inspect the arm in good light
    - i. Erythema should not be measured.

- ii. Look for induration (hard, dense, raised formation).
      - iii. Palpate the induration in all 4 directions to locate the margins or edges of induration
    - b. Measure induration
      - iv. Mark the edges with a dot. Palpate again to check if correct induration was marked.
      - v. If irregular- measure the longest diameter across the forearm.
      - vi. Measure from the one marked dot to the other marked dot (see Figure 1).
    - c. Record outcome
      - vii. In millimeters (patient file/ RTHC and study file).
        - 1. Do not record “non-reactive” or “negative” but record actual measurement.
      - viii. Record potential blistering.
    - d. Interpretation
      - ix. To be done together with clinical information and to be discussed with the study doctor/ PI.
10. Contraindications

**85. THE TUBERCULIN SKIN TEST SHOULD NOT BE ADMINISTERED TO:**

1. Patients known to be hypersensitive (Type I) to the active substance or any of the excipients. This can cause vesiculation and skin necrosis in the centre of a widespread tuberculin reaction. The necrosis will generally disappear after a few days.
2. Patients who previously have experienced a severe local reaction to Tuberculin products.
3. Those with documented active TB or a well-documented history of adequate treatment for TB infection or disease in the past. In such patients, the test is of no clinical utility.
4. Those with current major viral infections (e.g. measles, mumps, varicella).
5. Those who have received measles or other live virus immunization within the past 4 weeks, as this has been shown to increase the likelihood of false-negative TST results. Note that only measles vaccination has been shown to cause false-negative TST results, but it would seem prudent to follow the same 4-week guideline for other live virus immunizations – mumps, rubella, varicella (chickenpox) and yellow fever. However, if

the opportunity to perform the TST might be missed, the TST should not be delayed for live virus vaccines since these are theoretical considerations. (NOTE that a TST may be administered before or even on the same day as the immunizations but at a different site.)

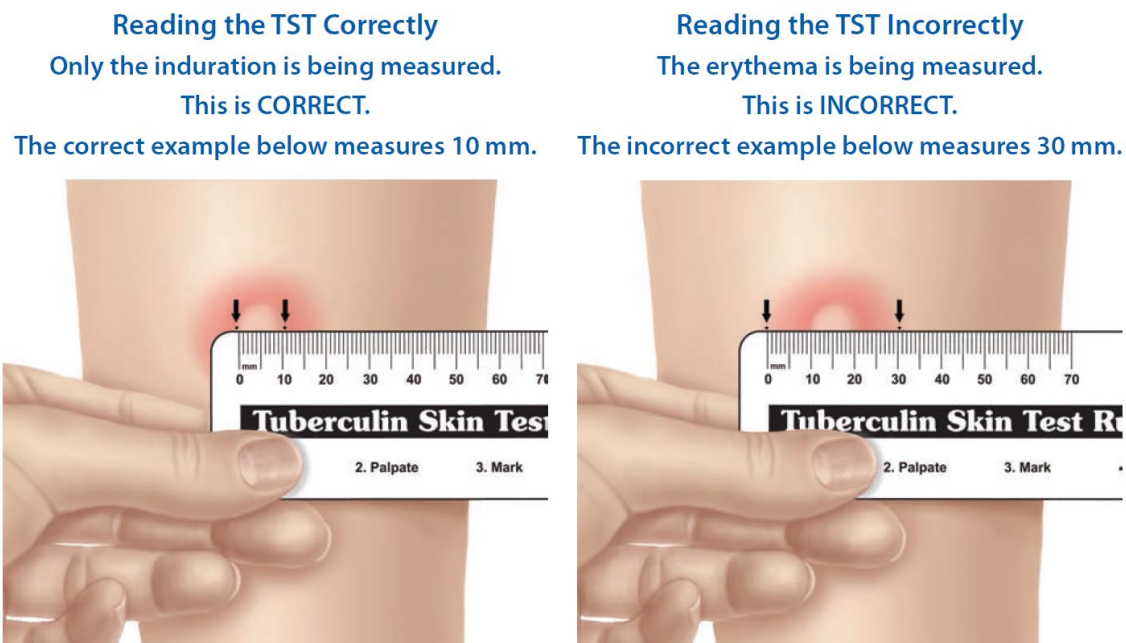
#### 86. SPECIAL WARNINGS AND PRECAUTIONS FOR USE

Although anaphylaxis is rare, facilities for its management should always be available during Mantoux tuberculin skin test.

#### Administering the Mantoux TST



**Figure 10.6. Administering the Mantoux TST (reference 5)**



**Figure 10.7. Reading the Mantoux TST (reference 5)**

## 87. REVIEW AND REVISION

This SOP will be reviewed annually by the Principal Investigator (PI) or designee.  
Refresher training is required every 12 months.

## 88. REFERENCES

1. Document based on CDC facilitator guide November 2013. Available at: <http://www.cdc.gov/tb/education/mantoux/pdf/mantoux.pdf>
2. WHO: Global tuberculosis control: WHO report 2014. Geneva, Switzerland: World Health Organization; 2014.
3. Marais BJ, Gie RP, Schaaf HS, et al. The natural history of childhood intra-thoracic tuberculosis: a critical review of literature from the pre-chemotherapy era. *Int J Tuberc Lung Dis.* 2004; 8: 392-402.
4. Menzies D, Pai M, Comstock G. Meta-analysis: new tests for the diagnosis of latent tuberculosis infection: areas of uncertainty and recommendations for research. *Ann Intern Med.* 2007; 146: 340-54.
5. Landi, S., et al.: Effect of oxidation on the stability of tuberculin purified protein derivative (PPD) In: International Symposium on Tuberculins and BCG Vaccine. Basel:

International Association of Biological Standardization, 1983. (Developments in Biological Standardization) 1986; 58: 545-552

6. CDC Core Curriculum on Tuberculosis: What the Clinician Should Know Sixth Edition 2013. Available at: <http://www.cdc.gov/tb/education/corecurr/>
7. Canadian Tuberculosis Guidelines 2013. Chapter 4, Diagnosis of latent tuberculosis infection. Available at: <http://www.respiratoryguidelines.ca/tb-standards-2013>

## 89. DOCUMENT REQUIREMENTS

Progress Notes

Source Document File.

## B.8 Standard Operating Procedure 8: Paediatric Chest

### Radiograph reading

#### 90. RATIONALE

Chest radiographs (CXR) form part of routine tuberculosis (TB) screening procedures in children where resources for these are available. For paediatric TB clinical research purposes, CXR are required in children with suspected pulmonary or extra-pulmonary TB disease. CXRs are reviewed by 2 experienced independent blinded reviewers and findings are described using pre-determined standard terminology on a standardized CXR Reporting Form. The rationale for the blinding to patient clinical data is that this limits potential interpretation bias. The aim of this SOP is to standardize procedures regarding the retrospective reading of CXRs by experts to ensure consistency in reading and reporting of paediatric CXRs for their classification on clinical trials.

#### 91. READING OF CXR'S

##### 1. Purpose:

Unless specifically stated otherwise in the study protocol / Manual of operations (MOP), the purpose of expert review of CXRs for clinical trials at DTTC is for retrospective classification of radiological findings in order to inform diagnostic certainty of TB disease, radiological pattern of pulmonary TB, radiological disease severity and radiological response to treatment. This process does not inform real-time clinical decision making. Therefore, this

process is additional to real time single review for clinical management and daily trial implementation purposes.

2. Timing:

CXRs are read retrospectively at times convenient to the expert readers. This should be done as timeously as possible, but is dependent on reader availability and resources.

3. Type:

Wherever possible digital images should be obtained, and be available for review on the hospital iSite PACs system. Where this is not possible (films taken at local clinics or Brooklyn Chest Hospital) hard-copy images are acceptable. Two views should be requested: antero-posterior (AP) and lateral. However, if no lateral film is available, readers can still continue to classify the radiograph. In older children PA films are preferred.

4. Reading equipment:

Digital CXR images should be viewed on the hospital iSite PACs system. Hard copy images should be reviewed on a good viewing box at all times.

5. Identification:

CXRs should be examined in a systematic manner, and the identity of the patient and the date of the CXR should always be checked prior to evaluation.

6. Quality:

The quality of the films is important, and should be evaluated by looking at 3 aspects: rotation, penetration and inspiration.

- a. Rotation is absent when the clavicle ends are the same distance from the midline, or if there is equidistance of the anterior rib ends into the lung fields.
- b. For penetration to be adequate, the inter-vertebral spaces should be just visible through the heart shadow.
- c. On adequate inspiration, 5-6 ribs should be visible anterior, and 8-9 ribs posterior above the level of the diaphragm.

If the reader decides that the quality of the CXR is insufficient for adequate interpretation, it should not be read and poor quality should be documented. This information should be captured on the CXR Reporting Form and a repeat CXR requested if still clinically appropriate.

7. Comparison with previous CXR:

Previous films should be available for comparison with follow-up CXRs where appropriate. Comparisons should be noted only after the follow-up film has been read fully using the CXR Reporting Form.

CXR Reporting Forms currently in use at DTTC, and referred to in this document include:

1. SDOC9 Standardized CXR Reporting Form
2. SHINE CXR Evaluation Forms (8A and 8B)

## 92. COMPLETING THE STANDARDIZED CXR REPORT FORMS

### 1. GENERAL GUIDELINES:

- a. Meticulous completion of all the required fields on the form is important.
- b. If the first reader interprets the CXR quality to be “unacceptable” (SDOC9 form Question A3 and SHINE form Question 2A), this should be marked in the appropriate field, and further completion of the form is not necessary, apart from signing and dating. Second reading does not need to be undertaken.
- c. If the reader interprets the CXR as “normal”, it is not necessary to complete further fields. The reader then only has to sign and date the form in the appropriate fields at the end.
- d. Once the reader has established that the film is “acceptable” quality and “not normal”, he / she can proceed to complete the rest of the form.
- e. All mandatory fields MUST be completed
- f. When complete, the form should be dated, RA code completed, and signed by the reader.
- g. Every form should be QC’ed by a DTTC clinician who is part of the relevant study team.

## 93. CLARIFICATIONS FOR SPECIFIC CXR REPORTING FORMS:

### 1. “SDOC9” CXR Reporting Form

- a. Section B:
  - i. Where ‘yes’ is marked, the relevant follow-on fields should ALL be completed
  - ii. Question ‘6’: only one option can be chosen (“1-Certain TB” OR “2-Uncertain TB” or 2-“Not TB”)
  - iii. If the classification of “1-Certain TB” or “2-Uncertain TB” is made, the TB disease classification (Section C- “Certain TB”) should be completed. If the classification of “3-Not TB” is made, an alternative diagnosis should be chosen and section D- “Not TB” should be completed. All applicable diagnoses should be circled (i.e. more than one option may apply).

- b. Section E
    - i. Question “1\_1” ‘Number of zones’ should correlate with the number of zones marked in the illustration for Question “1\_2”
    - ii. In Question “1\_3” extent = RUL (right upper lobe) refers to the total area of the lung parenchyma involved being equal size to the total area that is taken up by the RUL
  - c. Section F
    - i. Only completed where CXR being read needs comparison to a previous film
2. CXR REPORTING FORM
- a. Section ‘3A’ where “CXR typical of TB” option is chosen proceed to complete Sections 3B and 3D only (NOT Section 3C)
  - b. Section ‘3A’ where “CXR not typical of TB” is chosen proceed to complete Sections 3C and 3D only (NOT Section 3B)
  - c. Section ‘D’ should only be completed where CXR being read needs comparison to a previous film

#### 94. PRINCIPLE OF DUAL READING OF CXR'S

All CXRs should be read by at least 2 of the expert reviewers who have been appointed for this purpose at DTTC, using the appropriate standard CXR Reporting Form for the specific study.

Readers should have expertise and experience in interpreting paediatric CXR's in the context of tuberculosis, and should be blinded to clinical information and to each other's interpretation of the CXR findings.

If one reader classifies a CXR as “unacceptable quality”, this film does not have to be dual read. The CXR should first be repeated if this is still clinically indicated.

#### 95. CLASSIFICATION OF CXR FINDINGS FROM EACH INDIVIDUAL REPORTING FORM

Each individual CXR Reporting Form should be classified according to the following:

- 1. Diagnostic certainty
  - a. “SDOC9” CXR Reporting Form: 3 diagnostic options available:
    - i. “Certain TB” if **any** of the following fields are marked as ‘yes’



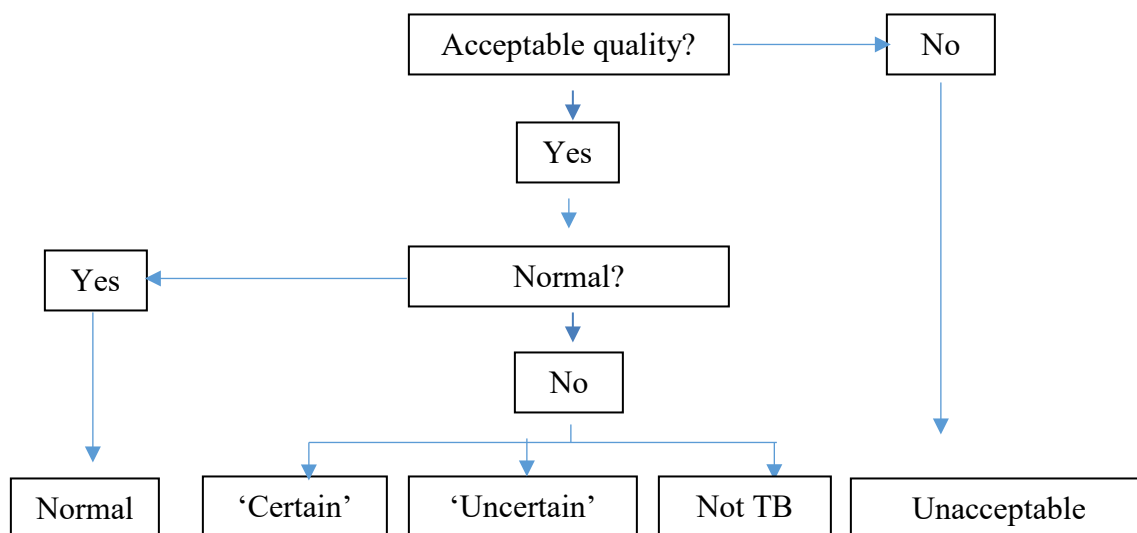
- Section B: Ghon focus / Expansile / Miliary / Perihilar nodes / Paratracheal nodes / Tracheal compression / Bronchial compression / Pleural effusion
  - Section B Question 6 marked “Certain TB”
  - Section C: Questions ‘1-9’
  - Section E: Question ‘2’
- ii. “Uncertain TB” if none of the “Certain TB” fields are marked, and the field Section B Question 6 marked “Uncertain TB”
  - iii. “Not TB” if none of the “Certain TB” or “Uncertain TB” fields are marked, and the field Section B Question 6 is marked as “Not TB”
- b. “SHINE” CXR Report Form: 2 diagnostic options available:
    - i. “Not normal, typical TB”
    - ii. “Not normal, not typical TB”

#### 96. RADIOLOGICAL PATTERN

1. “SDOC 9” CXR Report Form
  - a. More than one radiological pattern field can be marked
2. “SHINE” CXR Report Form
  - b. More than one radiological pattern field can be marked

#### 97. DISEASE SEVERITY

1. “SDOC9” CXR Report Form
  - a. There is no specific field on the form to capture radiological disease severity: classification by disease severity can be done if required by the specific study, and depending on which classification system is used
2. “SHINE” CXR Reporting Form
  - a. Form has a field for “Severe” versus “Non-Severe” disease. The algorithm below indicates the possible classifications of radiological diagnostic certainty for each individual “SDOC9” CXR Reporting Form



**Figure 10.8. Algorithm indicating the possible classifications of radiological diagnostic certainty**

**98. ESTABLISHING CONSENSUS BETWEEN FINDINGS FROM TWO INDIVIDUAL CXR REPORT FORMS**

Consensus criteria should be applied in order to obtain ‘consensus’ or ‘no consensus’ between the radiological findings noted by the two readers. In order to generate meaningful data that can be used in analysis and reporting, consensus needs to be obtained for the same categories as those used for each individual form:

1. Diagnostic certainty:
  - a. “SOC9” CXR Report Form:
    - i. the following consensus criteria should be applied
    - ii. when the outcome after two readers is ‘no consensus’ then a third reader is needed

**Table 10.9. Consensus table**

Normal	Certain TB	Uncertain TB	Uncertain TB	Certain TB	Certain TB	Not TB	Normal	Not TB	Not TB
--------	------------	--------------	--------------	------------	------------	--------	--------	--------	--------

Normal	Certain TB	Certain TB	Uncertain TB	Not TB	Normal	Uncertain TB	Uncertain TB	Normal	Not TB
↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
<b>Consensus 'Normal'</b>	<b>Consensus 'TB'</b>	<b>Consensus 'TB'</b>	<b>No consensus</b>	<b>No consensus</b>	<b>No consensus</b>	<b>Consensus 'Not TB'</b>	<b>Consensus 'Not TB'</b>	<b>Consensus 'Not TB'</b>	<b>Consensus 'Not TB'</b>

2. "SHINE" CXR Reporting Form

- a. the following consensus criteria should be applied
- b. when the outcome after two readers is 'no consensus' then a third reader is required

CXR normal	CXR typical of TB	CXR typical of TB	CXR typical of TB	CXR not typical of TB	CXR not typical of TB
CXR normal	CXR typical of TB	CXR not typical of TB	CXR normal	CXR not typical of TB	CXR normal
↓	↓	↓	↓	↓	↓
<b>Consensus 'Normal'</b>	<b>Consensus 'TB'</b>	<b>No consensus</b>	<b>No consensus</b>	<b>Consensus 'Not TB'</b>	<b>Consensus 'Not TB'</b>

- c. Consensus after two readers
  - i. For both "SDOC9" CXR Reporting Form and "SHINE" CXR Reporting Form, a third reader is required if there is 'no consensus' after two readers.
- d. Consensus after three readers
  - i. If two of three readers agree, the findings of the one discrepant reader should be discarded and consensus between the two readers who are in agreement should be taken
  - ii. If no consensus after three readers is obtained then a forum should be arranged for face-to-face discussion and joint decision regarding classification of the CXR.

99. RADIOLOGICAL PATTERN: FOR BOTH "SDOC9" AND "SHINE" REPORTING FORMS:

1. Consensus regarding radiological pattern should be sought only AFTER consensus regarding diagnostic certainty is obtained
2. For both “SDOC9” and “SHINE” CXR Reporting Forms:
  - a. Radiological pattern that is agreed upon by both readers should be taken as the ‘consensus’ pattern. Where some radiological pattern fields overlap but one or both readers have also marked additional fields, then the consensus field and most severe pattern should be reported (eg both readers mark “LN disease” and one also marks “miliary” – pattern should be reported as “LN disease and miliary”.
  - b. Where the two radiological patterns are completely discrepant, a third opinion should be obtained.

100. **DISEASE SEVERITY: FOR BOTH “SDOC9” AND “SHINE” FORMS:**

1. If the CXR is classified as ‘severe’ by ONE reader then it should be reported as ‘severe’, and should correlate with the radiological pattern

101. **REVIEW AND REVISION**

This SOP will be reviewed annually by the Principal Investigator (PI) or designee.  
Refresher training is required every 12 months.

## **B.9 Standard Operating Procedure 9: Tracing and retention of study participants**

102. **BACKGROUND**

Parents or caregivers should attend the scheduled clinic visits together with the study participant as per protocol requirements. The responsibility to attend the protocol-specified visits is counter-balanced by voluntary participation. The parent/caregiver may withdraw from the trial at any time without penalty or loss of benefits to which his/her child is entitled (ICH GCP 4.8.10).

When conducting a clinical trial on a treatment or intervention it is imperative to retain participants for the duration of the study and as outlined in the protocol. In the case of MDR-TB, child participants are typically followed for at least 18-24 months after enrolment. Retention of participants is vital as loss of participants equates to loss of data which

subsequently may cause trial delays and this may lead to regulatory submission delays and delayed launch of the product and lack of availability of necessary medicines for children. Participants' drop-outs are costly and pose a risk to the interpretation and validity of the research findings.

### 103. **OBJECTIVES**

This SOP describes the procedures to be followed for tracing parents/caregivers who have failed to attend a protocol-required visit and to maintain regular long-term contact with patients and their families to avoid loss to follow-up of participants.

### 104. **SCOPE**

This SOP applies to all DTTC CRS personnel as indicated on the cover page of this SOP.

### 105. **PROCEDURES**

#### 1. Baseline

##### a. Obtain as much relevant information as possible.

- i. Phone number: also neighbours'/grandparents' numbers. Obtain a minimum of 2 contact numbers (3 even better).
- ii. Address: do not simply copy from file/ record but let parent/caregiver confirm. Ask if she/he stays somewhere else often. Address of grandparents/boyfriend/crèche. Inform parent/caregiver that the study team might go to these alternative residences: obtain verbal permission to do so from him/her at initial contact and record this information in the source notes.
- iii. Document the participant's nearest primary care clinic (TB care and HIV care as relevant) and the clinic/hospital they usually visit.
- iv. If the participant lives in an informal settlement, try to determine how easy it will be to contact the participant. If resources are available, accompany the participant home after the initial visit, or agree on a convenient and nearby meeting point, from where the participant may be picked up for follow-up visits. Alternatively, make arrangements for the participant to be fetched from the nearest clinic on the day of follow-up.

Document how to get to the home with a map or landmarks, if an address is not sufficiently clear.

- b. Ask if the family has plans to travel outside the study area i.e. Cape Metropole and Western Cape Province (e.g. Eastern Cape province) during the period of follow-up and make plans accordingly.
  - c. Ensure that participants have the phone details of study staff allocated to them (e.g. DOT supporter, study coordinator) on their appointment cards and on their TB treatment cards.
  - d. Encourage participants (older children) or their caregivers to send a “Please-call me” in case they have no money to make a call to inform staff of their whereabouts or to reschedule appointments.
  - e. Obtain permission from parents or caregivers to contact their local TB and/or HIV clinic for the purpose of tracing and managing issues (e.g. concerns about treatment adherence).
2. Contact participants ahead of scheduled follow up visits as routine reminders to attend study visits.

Arrangements for follow-up visits should be made well in advance to the actual study visit, to allow ample time for location and rescheduling if needed.

a. Telephonic contact

- i. Try the number of the participant’s primary caregiver. Typically 3 attempts to the primary caregiver’s number (to be documented in source notes).
- ii. If not successful with the primary caregiver, try the other contact numbers once and document outcome of the phone call.
- iii. Documenting the outcome of phone calls:
  - Always document the date and time of the phone call (regardless of the outcome of the call); document the person spoken to and the plans made.
  - Not picking up the phone: Document this in the progress notes and try again another time of the day.
  - Voicemail: leave a message, clearly stating who you are and where you are phoning from. Document this in the progress notes.
  - Wrong number: document in progress notes.



- c. Confirm whether any traveling will be taking place between now and next visit and make plans accordingly.

#### 106. **IMPORTANT PRINCIPLES**

- a. Documentation must be done **on the day** of each tracing attempt.
- b. Write the contact tracing process down in the progress notes.
  - i. Date and time of tracing attempt and your name + RA code.
  - ii. Whom did you speak to?
  - iii. What was decided?
  - iv. If no contact was made, what was the next step planned and then done?
- c. Phone:
  - i. 3 times → on different moments of the day and on different days.
- d. Home visits:
  - i. Maximum 3 times → try also to plan on different moments of the day and different days.
  - ii. Be discrete about the study and why you are looking for participant and do not compromise patient confidentiality.
  - iii. Make sure we have an up-to-date list of addresses and phone numbers of participants at all times.

#### 107. **DEFINITIONS**

The tracing protocol must be followed **for every visit** for which the child is due, regardless of how many visits the child has missed before.

##### 1. Lost to follow-up (LTFU):

Each protocol will have a specific definition of how loss to follow-up is decided for that specific study. If, at the end of the window period for the last study visit there has been **NO** contact with the participant since the previous visit **AND** the whereabouts of the participant **ARE NOT** known, the participant may be declared **POTENTIALLY LTFU**. The protocol team should be consulted before this final assignment is made. Should the participant return after this date, follow-up and possible re-entry into the study may still be decided upon by the in consultation with the Protocol team. At the end of the study, the status of these participants will be re-assessed (withdrawn, unable to adhere to study requirements or loss to follow-up).

##### 2. Withdrawn from study:

If, at the end of the window period for the last study visit there has been no meaningful contact with the participant since the previous visit **BUT** the whereabouts of the participant



**ARE** known, the participant is declared **WITHDRAWN** by the study team at that moment. At the end of the study, the status of these participants will be re-assessed (withdrawn, unable to adhere to study requirements or loss to follow-up). This also applies to participants with whom there has been contact but who failed to attend the last visit within the window period as stipulated in the specific protocol. Please refer to each protocol for the definition of “withdrawn”.

3. Voluntary withdrawal from study:

If, at any point during the study the participant’s caregiver decides to withdraw from the study, the child will be withdrawn. This is termed a **VOLUNTARY WITHDRAWAL**. The necessary documentation needs to be completed. **NOTE:** No child can be declared LTFU or withdrawn/early discontinuation if the tracing protocol has not been followed **AND** documented. The protocol definition always takes precedence in such instances.

108. **REFERENCES**

None

109. **REVIEW AND REVISION**

This SOP is reviewed every two years (biennially) or earlier as required by the Sponsor, PI or designee.

## **B.10 Standard Operating Procedure 10: Principles of infection prevention and control**

110. **BACKGROUND**

The emergence of life threatening infections has highlighted the need for efficient infection prevention and control programmes in all health care settings. All study personnel could theoretically be exposed to communicable diseases. The risk varies depending on the pathogen and the type and degree of exposure. Infection prevention and control and occupational health and safety services work together to ensure the safety of patients, visitors and staff.

In our setting, the most important aspects to monitor and prevent are the transmission of hazardous biological agents like tuberculosis (TB) and blood-borne diseases (HIV, hepatitis

B and C), among others. There are several interventions designed to reduce the risk of infection transmission in healthcare including:

1. **Standard precautions** (previously called universal precautions) reduce the chance of infection transmission from both known and unknown (unrecognised) sources of infection. They protect healthcare workers, patients and staff from acquiring infection. Standard precautions should be applied to all patients in all circumstances, whether or not they are known to pose an infection risk. All healthcare workers should be trained in the application of standard precautions. Some key elements of standard precautions are:
  - a. Hand hygiene, Personal protective equipment, Safe injection practice and sharps management, Waste management, Patient placement (isolation), Cough etiquette (respiratory hygiene), Linen handling and segregation, Occupational health, Decontamination of equipment and the environment.
2. **Transmission-based precautions** are interventions put in place to reduce the chance of infection transmission for particular pathogens, e.g. airborne precautions for TB. They are always applied in addition to standard precautions.
3. **Procedure-based precautions** are the requirement for specific interventions to reduce risk of infection during a specified procedure.

**Table 10.10. The main routes by which micro-organisms can be spread (from reference 1)**

Main transmission	Types of transmission	Examples
Contact	Direct	Hands of healthcare workers
	Indirect	Equipment, e.g. thermometers, bedpans
	Sexual	Sexual transmission of HIV or syphilis
Respiratory	Droplet	Influenza, many other respiratory viruses
	Airborne (aerosols)	Tuberculosis, measles, chickenpox (varicella)
Ingestion	Water	Contaminated water, e.g. cholera
	Food	Contaminated food, e.g. salmonella
	Faecal matter (faeco-	Hepatitis A

Inoculation	Injection, trauma, surgery blood	Needlestick injury transmitting HIV, hepatitis B or C
	Insects / Vectors	Mosquitoes transmitting malaria
Transplacental	Mother-to-child	HIV, syphilis, rubella, etc

### 111. TRANSMISSION OF *M. TUBERCULOSIS*

*Mycobacterium tuberculosis* (*M. tuberculosis*) can be transmitted in virtually any setting. HCWs should be aware that transmission has been documented in health-care settings where HCWs and patients come in contact with persons with infectious TB. It is therefore necessary to have a TB infection control plan in place as part of a general infection control program.

The infectiousness of a TB patient is directly related to the number of droplet nuclei carrying *M. tuberculosis* (tubercle bacilli) that are expelled into the air (coughing, sneezing, speaking, singing etc). Depending on the environment, these tiny particles can remain suspended in the air for several hours. *M. tuberculosis* is transmitted through the air, and **not** by surface contact. Infection occurs when a person inhales droplet nuclei containing *M. tuberculosis*, and the droplet nuclei traverse the mouth or nasal passages, upper respiratory tract, and bronchi to reach the alveoli of the lungs.

In general, young children with pulmonary TB disease are **less** likely than adults to be infectious, because children are often unable to produce sputum when they cough, or may have paucibacillary TB. However, it is still possible for children to transmit *M. tuberculosis* to others if they have infectious characteristics, such as a positive AFB smear or cavity on a chest radiograph. In addition, the TB source case(s) for the infected child may be their primary caregiver or another household member who may accompany the child and therefore both children and their caregivers/visitors should be considered potentially infectious.

### 112. DECONTAMINATION

Decontamination is the process followed to ensure that re-usable medical devices are safe to use on the next patient (reference 1). Decontamination includes some or all of the following steps:

- Cleaning (physical removal of organic material including microorganisms)
- Disinfection (killing or destruction of most but not all disease-producing micro-organisms)
- Sterilization (destruction of all micro-organisms).

The method of decontamination is determined by the level of risk for infection transmission. For example, devices that will be in contact with the patient's bloodstream or sterile

tissue/sterile body cavities must be sterilized. Items that will only be in contact with intact skin, can undergo low-level disinfection, which should remove most pathogens. Alcohol and chlorine-based disinfectants are the most widely available. Depending on the concentration used, these chemical disinfectants can achieve low to intermediate level disinfection. Chlorine may not be suitable for all types of disinfection, as it can be corrosive (causing damage to metal surfaces). See reference 1 for more details.

### 113. OBJECTIVES

This standard operating procedures (SOP) is to provide health care workers at the research site with the tools to implement and adhere to appropriate infection control measures to protect themselves and others from transmission of potential infections.

### 114. SCOPE

This SOP applies to all personnel with direct patient contact, or exposure in waiting rooms and corridors or other relevant spaces (in the case of air-borne pathogens).

### 115. PROCEDURES

#### 1. Baseline Health Assessment Questionnaire

This part is under the responsibility of DTTC and SU Human Resources and Occupational Health. It is recommended that all personnel working at the research unit must complete a Baseline Health Assessment Questionnaire (Refer to the Baseline Health Assessment questionnaire from Stellenbosch University) when newly appointed (See also SOP AM006 Occupational Injury and Needle stick Injury Management).

- a. **Tuberculosis assessment** (see point 4.2)
- b. **Blood-borne pathogens (Hepatitis B, Hepatitis C and HIV):** Refer to SOP AM006 Occupational Injury and Needle Stick Injury Management
- c. **Influenza vaccine:** All personnel are encouraged to get the yearly influenza vaccine offered free of charge by DTTC.

#### 2. Tuberculosis assessment for personnel

- a. **Tests for TB infection:** TB tests (Heaf, Tuberculin skin test (TST), Mantoux, PPD or QuantiFERON) – to assess *M. tuberculosis* infection status will be done for all new employees, unless already positive in the past (see SOP on Tuberculin Skin testing).

- b. **Chest X-ray:** All newly appointed personnel will go for a baseline chest X-ray (CXR) paid for by the DTTC as part of standard HR procedure and using standard private healthcare providers, prior to starting work duties. If later on there are indications of new symptoms suggestive of TB, the new X-ray can be compared to this baseline X-ray to identify changes that might help to diagnose or exclude a condition.
- c. **Annual TB screening:** The DTTC has a standard policy on baseline and annual TB screening which will be followed. All personnel will be required to complete a yearly symptom screen checking for the above symptoms and submit it to the unit Infection Prevention and Control officer, who will be responsible for ensuring compliance. The form is the DTTC Annual TB Screening Questionnaire (25.06.2014) – see Appendix A
- d. **Symptoms:** The following symptoms are suggestive of TB and should trigger further investigation.
  - i. Persistent cough (2 weeks or longer);
  - ii. Chest pain;
  - iii. Bloody sputum;
  - iv. Weight loss or loss of appetite;
  - v. Fever;
  - vi. Chills;
  - vii. Night sweats;
  - viii. Malaise/Fatigue.
- e. **TB Evaluation:** Personnel having any of symptoms suspicious of TB should notify their line manager as soon as possible. The appropriate medical evaluation will be facilitated, which may include sputum analysis and chest radiography depending on the symptoms.

### 3. Personal Protective Equipment (PPE)

#### a. Respiratory-Protection Controls (Appendix B)

Personal protective equipment should be used in situations that pose a high risk for exposure to TB disease. They are the first line of defence, along with hand hygiene, in preventing the spread of infection from person to person.

#### b. N95 respirator masks for personnel (Appendix B)

N95 respirators are designed to protect HCWs and other at risk individuals from inhaling droplet nuclei as they filter the air before the person wearing the respirator inhales it. They provide a minimum of 95% filtration efficiency for particles 0.1-10 microns in size. The N95 respirators must be National Institute for Occupational Safety and Health (NIOSH) approved. Respiratory protection i.e. N95 respirators should be worn:

- i. By personnel when direct exposure to respiratory secretions are unavoidable e.g. sputum inducing procedures or nasogastric tube insertion, regardless of whether the patient is considered potentially infectious.
  - ii. Whenever aerosolisation or splattering is likely to occur.
  - iii. By personnel (N95) during transport of persons with potentially infectious TB.
  - iv. Masks do not need to be worn outside.
- c. N95 respirator fit-testing, use and storage
- i. All new personnel must be trained on how to wear the N95 respirators: two polyurethane headbands secure the mask to the face to help ensure a tight facial seal and prevent leakage around the mask edges.
  - ii. In order to be effective, it is essential that N95 respirators are properly fitted to the size and shape of an individual healthcare worker's face. Each personnel member must be fit-tested for respirator use at least every 2 years (a log of this testing is kept on record).
  - iii. N95 respirators are available in different sizes and styles, e.g. cone- or cupshaped; duckbill-shaped and others with built-in expiratory valves. This is to maximise the chance of finding a respirator that can provide a tight seal on the wearer's face.
  - iv. It is important to note that excessive facial hair or beards will prevent a proper seal being formed.
  - v. Each personnel member should use their own respirator (according to the respirator shape and size that they were fit-tested for).
  - vi. A single respirator can be re-used for up to 1 week or up to 8 hours of continuous use (whichever comes first) and then discarded in a clinical waste bin.

- vii. The respirator should be stored in a PAPER envelope (NOT a plastic bag as condensate/moisture will damage the filters).
- viii. Do not touch the inside of the mask with potentially infected gloves.
- ix. Discard any mask that has become moist or wet.
- x. Personnel should take care not to fold or crumple the respirator as this will damage the filters.

d. Surgical masks for patients (Appendix B)

Surgical masks are designed to “contain the cough particles” or reduce the number of droplets being exhaled into the air by persons when they breathe, talk, cough, or sneeze. A surgical mask worn by TB patients helps to prevent the release of TB aerosols from the person’s airway into the environment. Persons with potentially infectious TB disease should be given, and encouraged to use, a surgical mask to minimize the risk of expelling droplet nuclei into the air. Persons with potentially infectious TB will be considered any child or caregiver/visitor with any of the following:

- i. AFB smear or culture positive pulmonary TB
- ii. Not yet on effective TB therapy (i.e. untreated, on treatment for DS-TB for less than 2 weeks or on treatment for DR-TB for less than 2 months)
- iii. Non-adherent to TB therapy
- iv. Anyone actively coughing
- v. Anyone being investigated for possible active TB

For the Brooklyn Chest Hospital Unit, more details on this are described in SOP CM008 Patient Flow at PK Unit

e. Gloves

- i. Disposable non-sterile gloves should be worn to provide a protective barrier and to prevent cross contamination when performing phlebotomy, touching blood, body fluids, secretions, excretions, mucous membranes, and non-intact skin.
- ii. Gloves reduce the likelihood that microorganisms will be transmitted to patients.

- iii. Wash hands before putting on gloves. Gloves are to be removed immediately after contact, followed by proper hand hygiene (See Hand Hygiene SOP CM003)
- iv. Personnel must not wear the same pair of gloves to care for more than one patient.
  - v. Cover cuts or abrasions with waterproof plaster before putting on gloves.
  - vi. Disposable gloves should be worn for all cleaning purposes.
  - vii. Utility gloves for housekeeping purposes may be decontaminated for reuse provided that the integrity of the glove is not compromised. Utility gloves will be discarded if they are cracked; peeling, torn or exhibit signs of deterioration or the function of barrier is compromised.
- viii. Personnel with latex allergies should wear the non-allergic gloves.

f. Protective Eyewear

- i. Wear protective eyewear or goggles to protect mucous membranes of the eye when conducting procedures that are likely to generate splashes of blood, body fluids secretions or excretions.
- ii. Disposable eyewear must be discarded immediately. If they are reusable, decontaminate them according to the manufacturer's instructions.

g. Plastic Aprons

- i. Aprons must be worn as a waterproof barrier if contamination of clothing is likely to occur.
- ii. If a plastic apron is soiled, remove immediately without touching the outside and discard in clinical waste bin.
- iii. Cleaners should wear plastic aprons at all times when cleaning and handling medical waste. Plastic aprons should not be reused even though they may appear clean.

h. Lab Coats

- i. Only laboratory personnel are recommended to wear a lab coat when processing samples in the laboratory area on site. Laboratory coats should not be worn outside the processing/laboratory area.

i. Fluid shield / splash guard

- i. It is recommended that personnel that process laboratory samples make use of a fluid shield procedure mask with a wraparound splashguard



visor. This offers the protection of breathable film, which helps protect personnel against exposure to blood and body fluids.

- ii. In cases where study participant presents with or develops bleeding or haemoptysis: Use gloves, mask and apron and if haemoptysis is present, eye protection (visor/goggles). Place gauze on the site and apply pressure and maintain until the bleeding subsides. In cases of resuscitation: Use gloves, apron and mask and eye protection (visor/goggles).

#### 4. Prevention of needle stick and injuries from other sharp instruments

##### a. Procedure for phlebotomy

- i. Wear disposable gloves.
- ii. Ensure that sharps containers are available and not over-full. These containers should be easily accessible.
- iii. Ensure adequate lighting.
- iv. Try to ensure calm surroundings and prepare the study participant adequately in order to minimize struggling.
- v. Put EMLA or an appropriate alternative cream on selected puncture sites as per phlebotomy SOPs. Obtain adequate assistance if required.
- vi. If phlebotomy was performed with a needle on a syringe, place the specimen collection tube on a flat surface and place the needle on the stopper without touching or holding the tube. Once the needle is on the stopper the tube may be steadied. Do not hold the specimen collection tube whilst placing the needle into the stopper.
- vii. In cases where specimen collection tubes do not have stopper caps, use the sharps container needle removal device to remove the needle before filling the tubes from the syringe.
- viii. Never recap any needles/sharps.
- ix. Discard all sharps whole into the container immediately after use i.e. do NOT separate the needle and syringe before disposal unless needed to decant blood into specimen tubes.
- x. Mop up all blood on surfaces and disinfect these areas with 70% alcohol solution or sodium hypochlorite (bleach) solution at a strength of 10000 parts per million (PPM).

- xi. Remove gloves and perform hand hygiene (alcohol hand rub or hand wash- see details in SOP CM003).

b. Procedure for handling all needles:

- i. Gloves must be worn at all times.
- ii. When taking blood or working with blood, stay focused. Do not look away from the needle if someone speaks to you.
- iii. Needles must be disposed of in the biohazard sharps container only. No needles may be disposed of in a dustbin.
- iv. No needles may be re-sheathed after a blood draw.
- v. Once the needle is removed from the vein:
  - o Immediately discard the needle/butterfly and syringe directly into the appropriate biohazard sharps container.
  - o If unable to discard immediately, place the syringe and needle temporarily into a kidney bowl and discard as above as soon as phlebotomy procedure is finished.

c. Procedure for handling sharps

- i. Gloves must be worn at all times.
- ii. If a specimen container has broken, the pieces must be discarded into a biohazard sharps container.
- iii. When a sharps container has reached the  $\frac{3}{4}$  line on the box, it is sealed by closing the lid and clicking it shut.
- iv. Sharps containers must be available near areas where phlebotomy is done but out of the reach of patients (either mounted on the wall or kept on a hospital trolley).

## 116. RESPIRATORY HYGIENE AND COUGH ETIQUETTE

Respiratory hygiene is the recommended method for preventing transmission of respiratory illness, particularly in health- care settings (see Appendix C – Cough Etiquette)

Apply the following measures:

1. Cover your cough/sneeze with a sleeve or tissue. Never cough or sneeze into your hands.
2. Dispose of used tissues in garbage.

3. Wash hands after coughing or sneezing
4. Wear a surgical mask if you have influenza-like symptoms (cough and fever) or active TB disease. Use of general items, e.g. cutlery or books need not be limited as contamination of objects with respiratory secretions is not associated with the transmission of TB.

## 117. **TB INFECTION CONTROL**

### 1. Administrative Controls

- a. A TB infection control risk assessment of the facility is conducted annually (see SOP CM006).
- b. Properly clean, sterilize, or disinfect all the rooms as well as all equipment that might be contaminated (see SOPs LM002 and GC006).
- c. Education, training, and counselling of patients/study participants and visitors about TB infection and disease should be ongoing.
- d. Education, training, and counselling of HCW and study personnel with regards to TB and TB-IPC at recruitment (e.g. complete the TB-IPC self-study chapter (see reference below) and sign this SOP).
- e. Testing and evaluating HCW who are at risk for exposure to TB disease (baseline CXR, symptom screen and evaluation when symptomatic).
- f. Posters/signs regarding proper cough etiquette are posted in all clinical areas (Appendix C).
- g. Provision of tissues and surgical masks to coughing patients/study participants/caregivers.
- h. Screening for active TB and drug-resistant TB among patients/study participants and caregivers (obtain bacteriological results prior to study visits or entry of the facility).

### 2. Environmental Controls

- a. Environmental controls prevent the spread and reduce concentration of infectious droplet nuclei through ventilation technologies (natural ventilation or mechanical ventilation), and ultraviolet germicidal irradiation (UVGI).
- b. The Brooklyn Chest Hospital (BCH) Research Unit and the Khayelitsha Site C Research Unit are equipped with exhaust ventilation in all clinical and patient

waiting areas, which facilitates air exchange in the units and creates a negative pressure environment (to limit potentially contaminated air from passing into non-clinical areas of the units).

- c. Windows and doors in the clinical and patient waiting areas should remain closed so that these air extractors can function as designed.
  - d. Adequate functioning of the ventilation system should be determined monthly (holding soft tissue or a lit incense stick below ventilation outlet and checking proper air suction to the outside). Result must be recorded in a ventilation log by the on-site infection control officer or delegate (Appendix D).
  - e. The ventilation system should be serviced and maintained by a ventilation engineer annually or as required.
3. Collection of Respiratory Specimens (expectorated sputum, induced sputum and gastric aspirates)
- a. Outside (open air) space or a sputum booth located outside are the preferred spaces for obtaining expectorated sputum specimens and should be used wherever possible.
  - b. For procedure rooms/cough rooms, the door should be locked or a sign put on the outside indicating that specimen collection is taking place inside, to avoid anyone entering the room during the procedure.
  - c. All staff responsible for collecting respiratory specimens in the procedure rooms/cough rooms and performing gastric aspiration or lavage must wear personal protective equipment during throughout the procedure.
    - i. The personnel member collecting the specimen must wear a N95 respirator and disposable gloves.
    - ii. In addition, a plastic apron should be worn for the procedure if contact with mucous or respiratory secretions is anticipated.
    - iii. Caregivers assisting the staff with procedures (e.g. holding a child during specimen collection) should also be given particulate respirators to wear.
    - iv. All personal protective equipment (masks, gloves, and aprons) should be discarded appropriately after completion of the procedure.
  - d. Hand hygiene should be performed before and after participant contact (see SOP CM003 on hand washing).

- e. If the room provides at least 12 air changes per hour (ACH), the room must not be used for at least 35 minutes after the infectious patient has left the room, to allow enough air changes before another patient enters the room (see Table below from reference 7).
  - i. During this period, staff remaining in the room should continue to wear a N95 mask.
  - ii. A fake clock with moveable hands should be fitted on the outside of the door, to mark the time when the room can next be used after specimen collection has been carried out. Allow at least 10 minutes after specimen collection, before exiting the room. Close the door immediately. Indicate the time when the next procedure can be done, on the fake clock on the door.
- f. After collection, specimen containers should be closed tightly and the outside of the container should be disinfected before it is labelled. The sample is then placed in a sample bag. All specimen bags should be placed in appropriate transport boxes at the appropriate temperature for transport to the laboratory.
- g. All surfaces should be cleaned before and after each procedure with appropriate disinfectant (see SOP CM006).

**Table 10.11. Time required to clear the air of *M. tuberculosis* after the source patient leaves the area or when aerosol-producing procedures are complete (from reference 7).**

ACH	Minutes required for removal efficiency <sup>†</sup>	
	99%	99.9%
2	138	207
4	69	104
6	46	69
12	23	35
15	18	28
20	14	21
50	6	8
400	<1	1

\* This table can be used to estimate the time necessary to clear the air of airborne *Mycobacterium tuberculosis* after the source patient leaves the area or when aerosol-producing procedures are complete.

<sup>†</sup> Time in minutes to reduce the airborne concentration by 99% or 99.9%.

4. Transportation of patients and/or caregivers
  - a. Persons with potentially infectious TB (see above) should be transported in the back seat of a car fitted with a custom made screen between the front and backseats, if feasible.
  - b. If a car with a screen is not available, the participant or caregiver should wear a surgical mask and the driver should wear an N95 respirator.
  - c. For known MDR or XDR infectious patients, health care workers should use a N95 respirators (driver and nurse) and the patient should wear a surgical mask during transport.
  - d. During transport:
    - i. The driver puts his fan on to get air from outside (**not on re-circulated air option**)
    - ii. If possible, the driver should drive with rear windows open to allow the flow of air to the back and outside of the car.
  - e. After each transport:
    - i. After dropping the patient at destination, the driver must keep the fan on to get air from outside (not on re-circulated air option) and drive with rear windows open to allow adequate ventilation between patients.
    - ii. Wipe the screen with a disinfectant (this is to prevent the transmission of other pathogens such as respiratory viruses).
    - iii. Cars should be cleaned weekly.
5. Transportation of specimens
  - a. See specific instructions per protocol
6. Visitors
  - a. Close association of new contacts, especially if immune-compromised, should be avoided until the patient is deemed to be non-infectious.
  - b. Visitor will in general be limited to parents or other primary caregivers, and other immediate family members
  - c. Visitors will be required to adhere to the unit's infection control procedures
  - d. All visitors should be screened for TB by asking about any possible TB symptoms and TB treatment.
  - e. Visitors with TB or with potentially infectious TB disease should be limited, where feasible. Close family members with TB (parents/caregiver) will not be

discouraged from visiting their child but infection control precautions need to be adhered to.

#### 118. **PATIENT CARE EQUIPMENT**

All equipment must be cleaned after use and immediately whenever noted to be visibly soiled. The appropriate method of cleaning should be applied to ensure proper decontamination of equipment used for a patient.

1. General guidance:
  - a. Always wear gloves when cleaning patient care equipment.
  - b. Aprons should be worn by when cleaning soiled equipment.
  - c. All tape and tape residue must be removed from any equipment before cleaning.
  - d. Grooves on equipment may require cleaning with a cleaning brush.
  - e. Handle patient care equipment soiled with blood, body fluids, secretions and excretions with care to prevent exposure to skin, mucous membranes, clothing and the environment.
2. Cleaning of reusable equipment:
  - a. Stethoscopes should not be immersed in liquid but wiped down with a 70% alcohol solution.
  - b. Ear specula can be removed, washed with soap and water and wiped down with 70% alcohol and dried.
  - c. Blood pressure cuffs and pulse oximeters - probes can be wiped down with 70% alcohol solution or swabs and left to dry before reuse or stored.
  - d. Thermometers must be wiped with 70% alcohol and left to dry before storage or reuse.
  - e. Non-reusable suction catheters and nasogastric tubes as well as wooden spatulas must be discarded in the biohazard waste box.
  - f. Suction bottles must be emptied, washed with soap and water, wiped over with 70% alcohol and left to air dry.
  - g. Kidney dishes used for receiving specimen tubes or soiled swabs etc. should be washed with soap and water, dried and wiped over with 70% alcohol and left to air dry. Never use D-germ for cleaning equipment or surfaces – D-germ contains emollients/moisturizers and is used for hand decontamination only.

- h. Scales and measurement tools must be sprayed with 70% alcohol and dried between participants and properly cleaned and disinfected when soiled and at the end of the day.
- i. Surface disinfection is not routinely required – only where aerosol inducing procedures were performed: – first use soap and water then when dry, wipe over with 70% alcohol. On all other surfaces including floor, regular liquid soap and water.
- j. Any damaged equipment must be reported for decision on repair or replacement.
- k. Cables must be wiped thoroughly and dried when cleaning the apparatus.
- l. Gloves should be worn when handling soiled linen.

#### 119. **HANDLING BIOLOGICAL SPILLS**

The laboratory area and each DTTC-SU Vehicle used to transport study participants should have a biological spill kit on hand to effect a clean-up and decontamination of the spill area. All spills involving biological materials (blood, vomit, urine, feces, and respiratory specimens) should be treated as potentially hazardous.

1. After a spill has occurred:
  - a. Treat all biological spills with caution – cordon off/close the affected area to prevent additional human and environment contamination
  - b. Exercise extreme precautions with contaminated sharps like broken glass, microscopic slides, scalpels, etc
  - c. Keep gloved hands away from eyes, mouth and nose.
  - d. Wear eye protection and a surgical mask to protect against anticipated aerosol release from spills, splashes or sprays of infectious materials to the face. Prime entry routes for infectious agents are the mucous membranes.
  - e. Wear a protective disposable gown.
  - f. Wear disposable gloves to protect hands from potentially infectious materials or contaminated surfaces of equipment. Wear two pairs of gloves if necessary. Discard when contaminated, or remove when clean-up work is completed and discard. Wash hands thoroughly before leaving the laboratory.



- g. Wear appropriate disposable shoe covers to protect street shoes if the spill is on the floor. Never wear open-toed sandals when working in the laboratory.
- h. Notify the site safety officer and the central DTTC health and safety officer (if required); complete a report of the event.

## 120. REFERENCES

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## 121. DEFINITIONS

None

## 122. REVIEWS AND REVISION

This SOP will be reviewed every year or earlier as required by the Sponsor, the PI or designee.

123. **APPENDICES**

Appendix A - DTTC Annual TB Screening Questionnaire

Appendix B - Types of masks: N95 respirator for HCW and surgical mask for patient

Appendix C - Cough Etiquette

Appendix D - Ventilation Monitoring Log

Appendix E - Biological Spill Kit

## 124. APPENDIX A - DTTC ANNUAL TB SCREENING QUESTIONNAIRE

Revised by JV Arendse\_20140625

**TB SCREENING QUESTIONNAIRE ( 2014 )**

Name : .....

Date: .....

Staff number: .....

Position: .....

**Please fill in the table below. Mark with a X**

TB Symptom Screen		
Cough for > 2 weeks	Yes	No
Weight loss > 1.5 kg in last month	Yes	No
Fever > 2 weeks (Temp 38 C or higher)	Yes	No
Drenching night sweats	Yes	No
Coughing up blood	Yes	No
Chest Pain	Yes	No

❖ If you have 3 or more of the above symptoms, we will arrange that you go for the necessary tests.

125. **APPENDIX B: TYPES OF MASKS**

**N95 respirators for protection of personnel**

N95 respirators filter out droplet nuclei or respiratory particles. DTTC will preferentially use the flat-fold or Duckbill type.



**Surgical masks worn by patients for protection of the environment**

Such masks retain droplets that are coughed out by patients with open TB. Masks for that purpose are cheap and do not need to fulfil stringent technical requirements. They normally have a square shape and are used once only and then discarded.

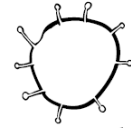
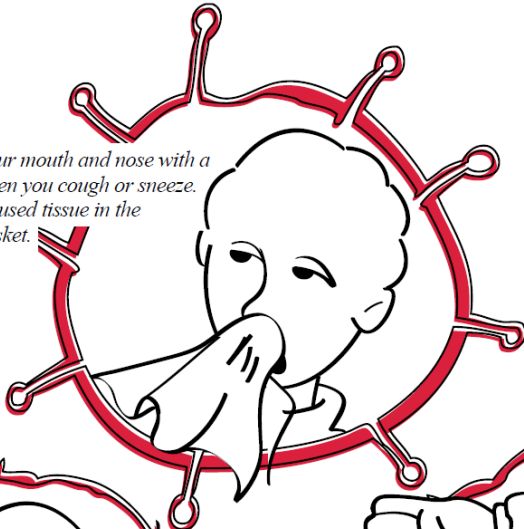


126. APPENDIX C COUGH ETIQUETTE POSTER

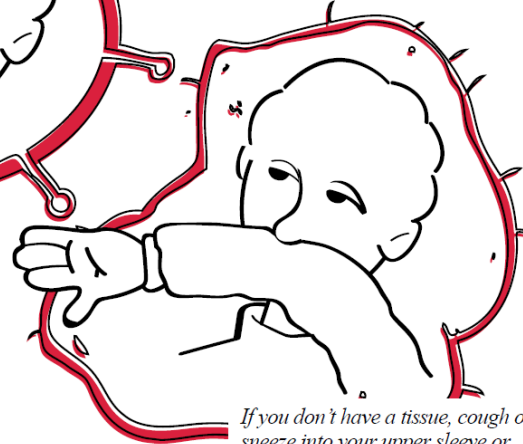
# Cover Cough

— Stop the spread of germs that can make you and others sick! —

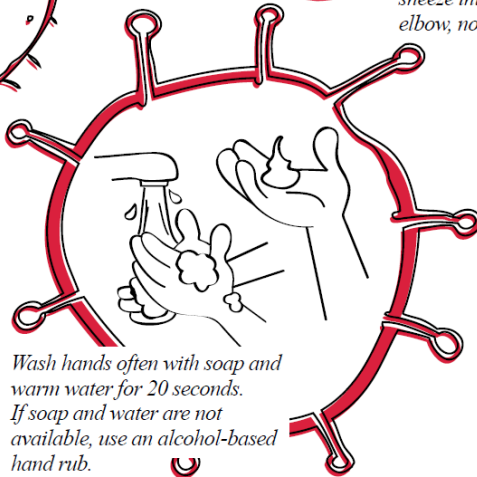
*Cover your mouth and nose with a tissue when you cough or sneeze. Put your used tissue in the waste basket.*



*If you don't have a tissue, cough or sneeze into your upper sleeve or elbow, not your hands.*



*You may be asked to put on a facemask to protect others.*



*Wash hands often with soap and warm water for 20 seconds. If soap and water are not available, use an alcohol-based hand rub.*

CS208322



## **Appendix E - The Biological Spill Kit**

The Biological Spill Kit should consist of the following items in a suitable plastic box:

- Household bleach (disinfectant to be diluted to 10%)
- A bottle of 70% alcohol (disinfectant)
- A two-litre plastic bottle for diluting the household bleach to 10% solution
- A spray bottle
- Sterilization bags
- Absorbent paper towels
- Yellow biohazard trash bags
- Disposable latex gloves
- A pair of metallic tongs
- Surgical masks
- Safety goggles, shoe covers and face mask
- Spill control and cleanup procedure

Except for the disposable items which should be replenished when used, the rest should be returned to the plastic box after use.

Examples of spill kit

[http://medicine.nus.edu.sg/medi/safetyweb/SOP\\_Handling%20of%20biological%20spills.pdf](http://medicine.nus.edu.sg/medi/safetyweb/SOP_Handling%20of%20biological%20spills.pdf)

<http://eclogistics.co.za/vehicle-compliance/spill-kits/>

### **General spill clean-up procedure**

For a spill of infectious or potentially infectious material, the following spill clean-up procedures should be used:

#### **Small spills:**

- a) Wear disposable gloves.
- b) Wipe spill area with paper towels soaked with disinfectant.
- c) Place contaminated paper towels in a biohazard bag for disposal.
- d) Repeat the procedure with fresh paper towels soaked in disinfectant.

#### **Larger spills:**

- a) Remove contaminated clothing and alert others in the vicinity of the spill and cordon off the area to prevent contamination of additional workers and environment

- b) Report the spill to the Safety Officer or Supervisor immediately.
- c) Identify any specific biological agent and assess degree of contamination and action required
- d) Allow aerosols to settle for 30 minutes
- e) Wear appropriate personal protective equipment (disposable gloves, lab coat, protective eyewear, shoe cover, etc)
- f) Cover spill with absorbent paper towels
- g) Carefully pour 10% bleach disinfectant or 70% Alcohol around edges of spill and then into the spill.
- h) Allow 20 minutes for the disinfectant to act on the spill.
- i) Wipe up the spill, working from the edges into the centre
- j) Pick up broken pieces of glass if any, and discard in a sharps bin.
- k) Clean spill area again thoroughly with fresh paper towels soaked in disinfectant
- l) Wash surface with soap and water and allow to dry.
- m) Place contaminated paper towels in an a biohazard waste container before disposal
- n) Remove used gloves and shoe covers, and wash hands thoroughly with soap and water.



## B.11 Standard Operating Procedure 11: Assessing anthropometric status in children

### 128. BACKGROUND

Anthropometry is the systematic measurement of the human body primarily focussed on dimensional aspects of body size and shape. These measurements are often used in resource limited settings, to diagnose malnutrition.

### 129. OBJECTIVES

This SOP describes the procedure to carry out systematic measuring of participants both in adult cases and paediatric.

### 130. SCOPE


Paediatric study teams at the Desmond Tutu TB Centre are required to be proficient in anthropometry.





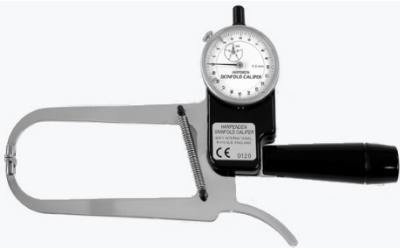
### 131. PROCEDURES

#### 1. Equipment:

Standardized calibrated measuring equipment will be used that will be available at each local study facility to ensure accurate anthropometric measures.

**Table 10.12. List of equipment**

Item	Description	Use
Paediatric/Baby Electronic Scale 	Must have an accuracy of 0.1kg	Weight measurement in infants (0-2 years)
Standing Electronic Scale	Must have an accuracy of 0.1kg	Weight measurement in participants older than 2 years

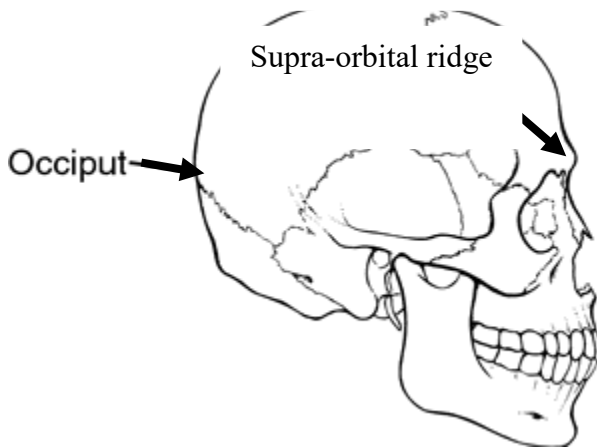
		
<p>Length board</p> 	<ul style="list-style-type: none"> <li>-Fixed head-board and moveable foot board.</li> <li>-Marked in millimetres</li> <li>-Made of a solid material</li> </ul>	<p>Recumbent length measurement in infants (0-2 years)</p>
<p>Stadiometer</p> 	<ul style="list-style-type: none"> <li>-Marked in millimetres</li> </ul>	<p>Height measurement in participants older than 2 years</p>
<p>Measuring Tape</p> 	<ul style="list-style-type: none"> <li>-Non-stretchable</li> <li>-Marked in millimetres and centimetres</li> <li>-Not wider than 1cm</li> </ul>	<p>Circumference measurement</p>
<p>Ball point pen</p>		<p>Used in mid-upper arm circumference measurement</p>
<p>Skinfold Calliper</p> 	<p>Harpenden calliper is considered to be the most accurate skinfold calliper and should be used for research purposes.</p> <ul style="list-style-type: none"> <li>-compression of 10g/mm<sup>2</sup></li> </ul>	<p>Skinfold thickness measurement</p>

	-marked in 0.2mm and 1mm	
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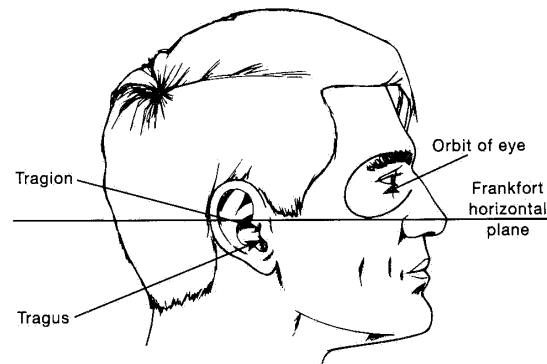
2. Measurements

a. Basic landmarks

OCCIPUT AND SUPRA-ORBITAL



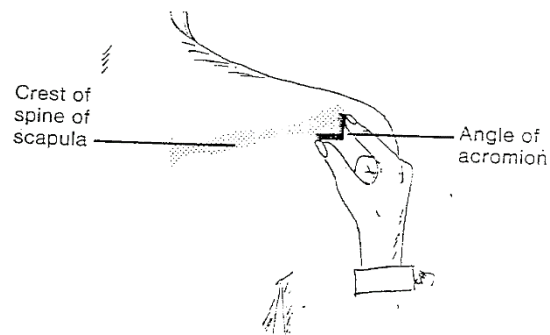
FRANKFORT PLANE



OLECRANON



ACROMION



**Figure 10.9. Basic landmarks**

3. General guidelines

- Wash hands before and after measuring a new subject
- Disinfect equipment before and after use
- Familiarize yourself with the equipment before measuring
- Where applicable, measure on the RIGHT hand side of the body

- e. Ensure safety of infants/children at all times
- f. Respect the privacy of the child
- g. Work quickly and involve caregiver to help soothe the child
- h. Two measurements must be taken and the average of these measurements is documented. If the two measurements differ substantially (>5-10%) then a third measurement should be taken and the median of the three used.

#### 4. Weight

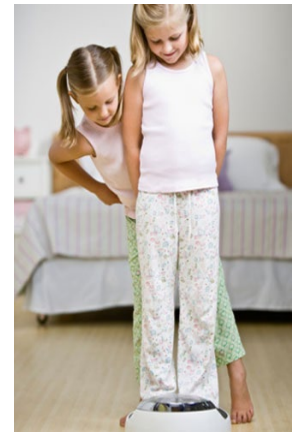
##### a. Infants < 2 years

- i. Place scale on flat hard surface
- ii. Place paper towel on scale
- iii. Undress baby completely and remove nappy
- iv. Turn on scale and ensure zero-calibration
- v. Place baby in the middle of the scale in sitting or lying position
- vi. The baby should not hold onto anything for support
- vii. Wait till the baby lies still before taking the measurement
- viii. Record the measurement to the nearest 0.1kg
- ix. Repeat



##### b. Children > 2 years

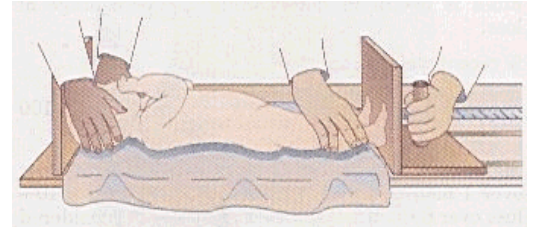
- i. Place the scale on a flat hard surface
- ii. Remove child's outer layers of clothing, diaper and shoes
- iii. Turn on scale and ensure zero-calibration
- iv. Let the child step onto the middle of the scale
- v. The child should not hold onto anything for support, let arms hang along the sides
- vi. Wait until the child stands still before taking the measurement
- vii. Record the measurement to the nearest 0.1kg
- viii. Repeat



#### 5. Length (infants < 2 years)

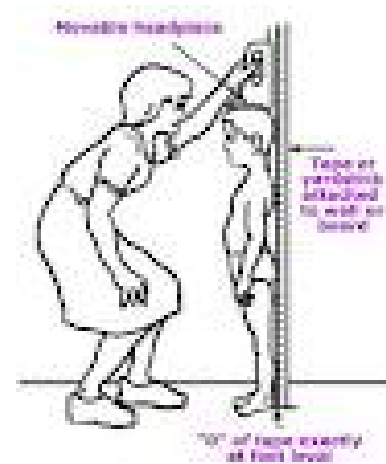
- a. Place length board on flat hard surface
- b. Ask the caregiver or a second person to help you with the measurement
- c. Remove infant's head coverings, shoes and socks

- d. Place the infant flat on his/her back on the length board
- e. Position the head so that the Frankfort plane forms a 90° angle with the back of the length board.
- f. The crown of the head should be touching the head board; ask the second person to hold this in position
- g. The shoulders and buttocks should be flat against the back board
- h. Stretch out the legs with one hand while you bring the foot board up against the heels of the feet (toes pointing upward). If it is impossible to get both legs straight then one leg may be used.
- i. Make sure the head is still in position before taking the measurement
- j. Record the measurement to the nearest 0.1cm
- k. Repeat



6. Height (children > 2 years)

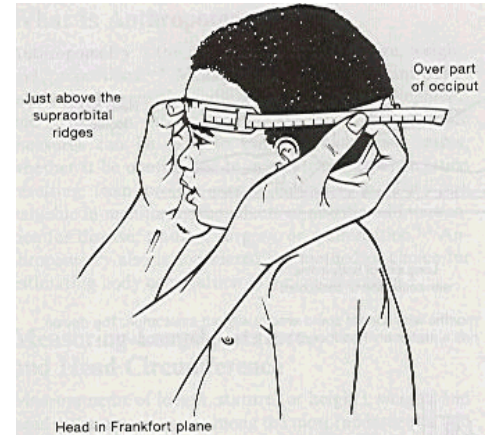
- a. Remove the child's shoes and socks and any head coverings
- b. Let the child stand upright with the back of their head, shoulder blades, buttocks and heels touching the length axis of the stadiometer
- c. Feet flat and legs together, arms hanging by the side
- d. Position the head so that the Frankfort plane forms a 90° angle with the length axis of the stadiometer
- e. Ask the child to take a deep breath or gently push in their tummy to help them straighten out
- f. Move the head board down until it touches the crown of the head and hold it in place



- g. Let the child step away from the stadiometer
- h. Record the measurement to the nearest 0.1cm
- i. Repeat

7. Head circumference

- a. Remove any objects covering the head
- b. When measuring a baby: Ask the caregiver or a second person to hold the baby upright with the head positioned so that the Frankfort plane runs parallel to the floor
- c. When measuring a child: Let them stand up straight and position the head so that the Frankfort plane runs parallel to the floor
- d. Place the measuring tape over the supra-orbital ridge and over the widest part of the occiput at the back of the head
- e. Make sure that the measuring tape is in a horizontal plane right around
- f. Pull the measuring tape tight around the head
- g. Record the measurement to the nearest 0.1mm
- h. Repeat



8. Mid-upper arm circumference

- a. Infants can be held by their caregiver or a second person in a seated position
- b. Older children can stand upright with their arms hanging by their sides
- c. Remove any clothing that is covering the child's arms so that the right arm is fully exposed
- d. Locate the midpoint of the arm:
  - i. Flex the arm so that the elbow is bent at a 90° angle with the palm facing upward

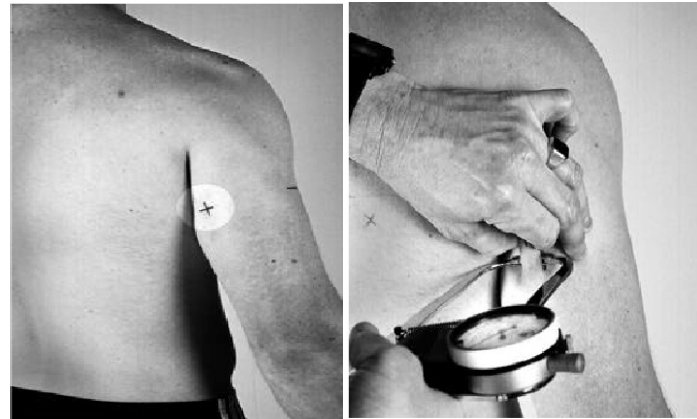
- ii. Measure the distance between the acromion of the shoulder and the olecranon process of the ulna, in the posterior position, and make a small mark with a pen halfway between those points.
- e. Take the circumference measurement:
  - i. Relax the subject's arm so that it is hanging by the side with the palm facing the trunk/thigh
  - ii. Place the measuring tape around the arm at the midpoint of the arm
  - iii. Make sure the tape is horizontal right around
  - iv. Make sure that the tape fits snugly, but that it does not cut into the skin
  - v. Record the measurement to the nearest 0.1cm
  - vi. Repeat



#### 9. Triceps skinfold

- a. Infants can be held by their caregiver or a second person in a seated position
- b. Older children can stand upright with their arms hanging by their sides
- c. Remove any clothing that is covering the child's arms so that the right arm is fully exposed
- d. Allocate the midpoint of the arm:
  - i. Flex the arm so that the elbow is bent at a  $90^{\circ}$  angle with the palm facing upward
  - ii. Measure the distance between the acromion of the shoulder and the olecranon process of the ulna, in the posterior position, and make a small mark with a pen halfway between those points.
- e. Mark of the skinfold site
  - i. The tricep skinfold site is allocated at the midpoint of the arm over the tricep muscle. Mark this site with a cross: vertical line over tricep muscle, horizontal line at midpoint of arm
- f. Take the skinfold measurement
  - i. Ensure that the skinfold calliper is zero-calibrated
  - ii. Using your thumb and index finger of your left hand, pick up the skin and subcutaneous fat tissue 1cm above the skinfold site mark
  - iii. The skinfold should run parallel to the long axis of the arm

- iv. Take the skinfold calliper in your right hand and place the calliper blades on the mark, halfway between the base and the top of the skinfold
- v. Hold on to the skinfold while releasing the calliper
- vi. Wait 2-3 seconds before reading the measurement
- vii. If the needle of the calliper goes beyond a whole turn, 20mm has been added to the reading.
- viii. Record the measurement to the
  - i. nearest 0.2mm
- ix. Repeat



132.

133.

134.

135.

136.

137.

138.

139.

#### 140. **SOP TRAINING**

##### 1. WHO Training Video

The WHO training video for paediatric anthropometry will be shown to enforce learning. This video is available at <http://www.who.int/childgrowth/training/en/> for future reference.

##### 2. The PI or designee arranges training on SOPs for:

- a. New and existing staff members
- b. Retraining on revised SOPs
- c. Retraining of a staff member in the event of a SOP violation/continuous SOP violations
- d. There are two mechanisms for SOP training: attendance at a formal SOP training session, and self-study.



- e. Once staff members have read/trained on a SOP, they must sign the signature log which is attached to each SOP as proof of training/self-study.
- f. These signature logs are kept in the study coordinator's office until all relevant staff have signed, after which it is filed together with the Master Copy in the office of the CRS Coordinator.

#### **141. REFERENCES**

None

#### **142. DEFINITIONS**

None

#### **143. REVIEW AND REVISION**

This SOP will be reviewed biennially, or as required by sponsor, the PI or designee.

## **B.12 Standard Operating Procedure 12: Obtaining informed consent or assent for children in clinical research**

#### **144. BACKGROUND**

Informed Consent\Assent - are processes by which an individual voluntarily expresses his\her willingness to participate in research, after having been informed of all aspects of the research that are relevant to his\her decision. Informed consent (IC)\assent is rooted in the ethical principle of respect for persons. It is not merely a form or a signature, but a process, with four key considerations:

1. Information exchange
2. Comprehension
3. Voluntary Participation
4. Documentation

The purpose of the IC/assent process is to protect the safety, rights and well-being of all study participants. IC\assent assures that prospective participants and/or their legal guardians understand the nature of the research and that they and their legal guardians can be knowledgeable and voluntarily decide whether to participate in the study.

#### 145. **OBJECTIVES**

The purpose of this standard operating procedure (SOP) is to outline a standard approach for obtaining written IC or assent and to ensure compliance with ICH - GCP and SA - GCP requirements.

#### 146. **SCOPE**

1. It is ultimately the Principal Investigator's responsibility to ensure that IC/assent is appropriately obtained and the process documented.
2. The PI can delegate responsibility of being involved in the process of obtaining IC\assent to other study team members, such as: counsellors, recruiters, study coordinators, nurses, clinicians and sub- investigators.
3. This SOP applies to all registered sites involved in clinical research studies at the DTTC.
4. Documents:
  - a. Informed Consent Form (ICF)\assent: this consists of a Participant Information sheet and a signature page.
  - b. The IC/Assent document must cover all aspects mentioned in the current ICH-GCP Guidelines
  - c. Informed Consent Checklist (ICC): this is a document used by study personnel to document that due process was followed in the consent taking.
  - d. The IC/Assent documents (and translations of these documents) must be approved (translations are acknowledged) by the Health Research Ethics Committee of the University of Stellenbosch (HREC-SU) and by the South African Health Products Regulatory Authority (SAPHRA) as required for the specific study, prior to implementation on site.
  - e. An expired consent/assent form should never be used. IC documents (new or updated) must be implemented on site as soon as possible after Ethics/Regulatory approval and on-site training (if required). Except for the master copy filed in the Site Investigator Files, all copies of superseded versions must be destroyed.
  - f. For DAIDS sponsored studies, all consent forms must be submitted to DAIDS for review. All consent forms for new protocols and amendments must be approved by DAIDS (see DAIDS SOP DWD-POL-CL-04.00 Requirements for Source

Documentation in DAIDS Funded and/or Sponsored Clinical Trials), prior to being implemented.

**147. PROCEDURES**

1. Protocol-specific consent/assent must be obtained prior to any study-directed procedure taking place.
2. Check that the written IC/assent document is the latest version approved by the HREC-SU, the South African Health Products Regulatory Authority (SAHPRA) and any other relevant local health authority (as required).
3. In the case of a minor (< 18 years), a parent or legal guardian must provide consent and this must be appropriately documented in the ICF checklist. The SOP for “Guidelines pertaining to legal guardianship” (SOP GC002) must be followed.

**148. PAEDIATRIC CLINICAL RESEARCH STUDIES**

1. Consent by parent or legal guardian
  - a. The parent or legal guardian signing written permission on behalf of the child, must be competent in terms of age (18 years and older), mentally healthy, have full capacity and compos mentis.
  - b. The parent/legal guardian is briefly introduced to the study and asked whether s/he would be interested in having their child/ward participate in the study.
  - c. If the parent/legal guardian expresses interest in participation:
    - i. Personnel delegated to be involved with IC will explain all the aspects of ICF with the participant.
    - ii. The parent/legal guardian is offered the option of reading and reviewing the ICF at that time, or taking the ICF home to review with friends and/or family members. S/he is then given the written ICF in the language that s/he is able to read and understand.
    - iii. The parent/legal guardian must be given the option to ask questions.
    - iv. A ICF checklist must be completed to indicate that the process was completed as per site SOP, i.e. whether questions were asked, and to document that the participant was offered the ICF to take home – see Appendix 1 (ICF checklist).

- v. The consent process must take place in a private environment thereby ensuring patient confidentiality.
  - vi. The consent discussion includes an explanation of all aspects covered in the ICF.
  - vii. The person obtaining consent assesses the parent/legal guardian's understanding of the contents and process of the IC. This may be done using a protocol specific comprehension test or it may be done ad hoc by the personnel taking consent and documented thoroughly in the ICF checklist.
  - viii. If required, the understanding will be verified by the PI/SI by means of documentation in the ICF checklist.
- d. The parent/legal guardian must fully agree to participate in this study. The participation agreement must be voluntary and without coercion.
  - e. It must be made clear what a part of the main study is and what is therefore compulsory if on the trial and what aspects are a part of sub-studies with purely research interests and may be opted-out of while remaining on the trial.
  - f. The parent/legal guardian must be informed that he/she can freely decide to withdraw his/her child from the study at any time without being penalized. Non-participation in the study will not affect their or their child's routine clinical care.
  - g. The ICF must be in the parent/legal guardian's home language or if he/she requests it to be in another language this should be documented in the ICF checklist. The parent or legal guardian should also write the name of the child and the child's date of birth on the ICF in their own handwriting.
  - h. If the parent/legal guardian expresses a desire to enroll the potential study participant, s/he is then asked to complete their own full name and surname, the full name and surname of the study participant and to sign and date the ICF himself/herself. Initials must not be used to sign. It should be explained to the participant/parent/legal guardian that he/she needs to sign by using her/his full names and surname as per DAIDS SOP DWD-POL-CL-04.00.
  - i. The parent/legal guardian must initial the bottom right corner of every page of the Patient Information Sheet and sign the signature page. If parent/legal guardian refuses participation in the study, the relevant study team members should document this on the ICF checklist and screening log.

- j. The person obtaining consent completes his/her full name and surname and signs and dates the ICF only after the parent/legal guardian has done so and the person obtaining consent is satisfied that the participant and/or parent/legal guardian understands the entire process. The time that the consent was obtained must also be captured on the ICF checklist so as to be able to verify that consent was obtained prior to any study related procedures were done.
  - k. The completed originally signed ICF is filed in the Investigator Site Files and two (2) copies are made and certified as true copies of the original. Note that QC must be done prior to certification. The certified copy means that another study team member will view the original document and verify that the copies made are an exact of the original by stamping the copies and signing and dating the stamp stating that this an exact copy of the original. One copy is given to the parent/legal guardian prior to initiating any study related procedure, the other copy is filed in the participant's study medical source file in accordance with SA GCP.
    - i. In order to protect these forms from over-handling, they must be filed as the last documents in the participant source folder.
    - ii. Further to this there must be a divider separating the ICF copies from the rest of the file.
  - l. The parent/legal guardian must sign acknowledgement of acceptance of their copy on the IC Checklist.
  - m. If the parent/legal guardian does not want to take the form, the copy meant for them is filed with the original ICFs and the checklist and comprehension (if used). A note will be made on the ICF checklist that the parent/legal guardian chose to leave it in the care of study personnel.
  - n. If a subsequent consent form (either a new version or a sub-study) needs to be signed, the participant and parent/legal guardian should be shown the copy of the previous consent form and asked to sign the same way if possible. If the signature differs from a previous signature, a verification note stating that they are both indeed the parent/legal guardian's signatures, should be completed and filed with the original ICF provided by the parent/legal guardian.
2. Assent by minor
- a. Although, the age of assent will be decided by HREC-SU, in general, if the child is 7 years or older they must be presented with a protocol specific and HREC

approved Assent Form to document their assent to be on the trial, unless the investigator decides that the child is not ready yet to understand the IC content adequately (decision must be clearly documented in the ICF checklist). The child should be given the necessary information in a child-friendly way to enable the child to express his or her opinion. The child's opinion must then be given "due consideration" in the decision-making process

- b. If the child refuses, this settles the matter and the child should not be enrolled, in accordance with SA GCP guidance. Failure to object to the trial is not the same as a child giving assent. There must be an affirmative agreement from the child.

#### 149. **ADULT CLINICAL RESEARCH STUDIES**

1. Study personnel delegated will attempt to establish the age of the potential participant using an identity document, birth certificate or letter from asylum seekers. Prior to the IC process, study personnel would have verified the participant's age by viewing the Identity document (or other relevant documents) and must ensure that a certified copy of the document is in the participant source document as evidence thereof. Note that QC must be done prior to certification. The certified copy means that another study team member will view the original document and verify that the copy made is an exact copy of the original by stamping the copy and he/she will sign and date the stamp stating that this is an exact copy of the original.
2. The potential participant is briefly introduced to the study and asked whether s/he would be interested in participating in the study.
3. If the potential participant expresses interest in participation:
  - a. Personnel delegated to be involved with IC will explain all the aspects of ICF with the participant.
  - b. The participant is offered the option of reading and reviewing the ICF at that time, or taking the consent form home to review with friends and/or family members. S/he is then given the written ICF in the language that s/he is able to read and understand.
  - c. The participant must be given the option to ask questions.
  - d. An ICF checklist must be completed to indicate that the process was completed as per Site SOP, i.e. whether questions were asked, and to document that the

participant was offered the consent form to take home – see Appendix (ICF checklist).

- e. The consent process must take place in a private environment thereby ensuring patient confidentiality.
- f. The IC discussion includes an explanation of all aspects covered in the ICF.
- g. The person obtaining consent assesses the participants understanding of the contents of the IC. This may be done using a protocol specific comprehension test or it may be done ad hoc by the personnel taking consent and documented thoroughly in the ICF checklist.
- h. If required, the understanding will be verified by the PI/SI by means of documentation in the ICF checklist.
- i. The participant must fully agree to participate in this study. The participation agreement must be voluntary and without coercion.
- j. It must be made clear what a part of the main study is and is therefore compulsory if on the trial and what aspects are a part of sub-studies with purely research interests and may be opted-out of while remaining on the trial.
- k. The participant must be made aware that he/she can freely decide to withdraw him/herself from the study at any time without being penalized. Non-participation in the study will not affect their routine clinical care.
- l. The ICF must be in the participant's home language or if he/she requests it to be in another language, this should be documented in the ICF checklist. The participant should also write their full name and surname on the ICF in their own handwriting.
- m. It should be explained to the participant that he/she needs to sign by using her/his full names as per DAIDS SOP DWD-POL-CL-04.00.
- n. The person obtaining consent completes his/her full name and surname and signs and dates the ICF only after the participant has done so and the person obtaining consent is satisfied that the participant understands the entire process. The time that the consent was obtained must also be captured on the ICF checklist document so as to be able to verify that consent was obtained prior to any study related procedures were done.
- o. The completed originally signed ICF is filed in the Investigator Site Files and two (2) copies are made and certified as true copies of the original, one copy is given

to the participant prior to initiating any study related procedure, the other copy is filed in the participant's medical record in accordance with SA GCP.

- i. In order to protect these forms from over-handling, they must be filed as the last documents in the participant source folder.
  - ii. Further to this there must be a divider separating the ICF copies from the rest of the file.
- p. The participant must sign acknowledgement of acceptance of their copy on the IC Checklist document.
- q. If the participant does not want to take the form, the copy meant for them is filed with the original ICFs and the checklist and comprehension. A note will be made on the ICF checklist that the participant chose to leave it at site in the care of study personnel.
- r. If a subsequent consent form (either a new version or a sub-study) needs to be signed, the participant should be shown the copy of the previous consent form and asked to sign the same way if possible. If the signature differs from a previous signature, a verification note stating that they are both indeed the participant's signatures, should be completed and filed with the original ICF provided by the participant.
- s. If participant refuses participation in the study, the relevant study team members should document this on the ICF checklist and screening log.

#### 150. **SPECIAL CONSIDERATIONS**

1. Consenting a participant or guardian who only speaks a language for which consent has not been translated (use a translator):
  - a. The study team will use HREC approved English or HREC-SU acknowledged translated consent form in a language chosen by the translator.
  - b. The translator must be fluent in the language of the consent document as well as the language of the participant or legal guardian.
2. Consenting a participant/guardian who is illiterate (use an impartial witness):
  - a. An impartial witness is required under the following circumstances:
    - i. If the participant/parent/legal guardian is illiterate
    - ii. If the participant/parent/legal guardian is unable to read or write due to a physical incapacity e.g. blindness.



- b. An impartial witness is present during the entire IC discussion. They are there to prove that the discussion covers the information in the ICF and that verbal consent was given by the participant/parent/legal guardian.
- c. The impartial witness is not a part of the study personnel and is someone of the participants/parent/legal guardian/s choosing or approval. They may be a member of the organization but they must not work on the study in question.
- d. The impartial witness must be fluent in the language(s) in which consent is being discussed.
- e. The impartial witness validates the IC discussion by reading through the written ICF and any other written information supplied to the participant in a language understandable to the participant/parent/legal guardian and verifying the accuracy and completeness of the verbal discussion.
- f. Illiterate persons may have the consent read to them in a language they can understand and “make their mark”. This may be a signature, or a thumb print.
- g. The impartial witness must initial the bottom right corner of every page of the Patient Information Sheet and sign on the last page at the dedicated spot.
- h. The impartial witness should enter the participant’s name and signature date near the space provided for the “participant’s printed name” and “signature date” on the ICF.
- i. The impartial witness must also complete their full name and surname, sign and date the ICF on the spaces provided for them.
- j. The person obtaining the IC should be the last person to initial the pages of the PIS, and sign and date the signature page.
- k. It should be documented in the consent checklist that the person cannot sign his/her name and that it is their “mark”.
- l. Study personnel should be cautious when enrolling participants who may not truly understand what they have agreed to participate in.

### 3. Consenting a primary caregiver:

Background from Ethics in Health research, second edition 2015, section 3.2.2.3. p 32:

“Many minors in South Africa do not have parents and very few have court-appointed guardians. These minors are often described as ‘orphans and vulnerable children’.”

It is ethical and reasonable to designate parental substitutes in these circumstances and get consent from primary caregiver.

Caregiver definition (Childrens' act) defined as "...any person other than a parent or guardian, who factually cares for a child and includes:

- a. a foster parent
- b. a person who cares for the child with the implied or express consent of a parent or guardian of the child
- c. a person who cares for the child whilst the child is in temporary safe care;
- d. the person at the head of a child and youth care centre where a child has been placed
- e. the person at the head of a shelter
- f. a child and youth care worker who cares for a child who is without appropriate family care in the community
- g. the child at the head of a child-headed household.

The guidance is premised on three conditions, all of which must be satisfied.

1. The risk standards set out minimum conditions for research involving minors (p 29 2<sup>nd</sup> edition Ethics in Health research 2015) must be adhered to; and
2. It is not possible to do the research with adult participants; and
3. The research proposes to investigate a problem of relevance to minors.

In other words, primary caregivers can be the ones signing consent for a study provided that there is no available legal guardian available, and the study poses minimal risk of harm or a minor increase over that (the degree of risk of harm should be justified by the risk-knowledge ratio). Ethical approval from Stellenbosch University institutional HREC will be sought on a case-by-case basis before enrolling any children with a primary caregiver only. For studies that involve observational, routine practices a general clearance letter can be submitted to get overall study approval for consenting primary caregivers for routine and observational practices only.

4. Consent for Permission for transportation of a study participants for research

Where it is foreseen that participants may need to be transported for study specific requirements (e.g. blood tests, ear examinations, or other tests for TB), separate consent for transport will be obtained – see SOP AM005.

#### 5. Stored Blood, Urine and Bacteriological Specimen Samples

If the study involves potential further tests that will be done on stored blood, urine and other samples, consent or written permission is also needed from the participant and/or parent/legal guardian. During the IC process the participant and/or parent or legal guardian must be made fully aware of this possibility and if the participant agrees, must give consent for storage and future use of samples and that they may or may not be (whichever is the case) informed of the test results. All information must be included in the written ICF. Either a separate consent form or a section of the informed consent form, for storage of additional or residual samples is required.

#### 6. Genetic Testing

The HREC SOP of Stellenbosch University (V4.3 dated June 2016) stipulates that the Participant Information and Consent document for genetic research must be separate from the main consent form.(see Section 12.2: Informed consent for genetic research of the HREC SU SOP V4.3 dated June 2016).

#### 7. Corrections made to ICFs and supporting documents

- a. Corrections are to be made by horizontally striking through the incorrect information (by the person who initially wrote it), then writing in the correct information as close as possible to it.
- b. The person making the correction must initial and date the correction and also write in the IC checklist the reason that information has been changed (if required and possible).

#### 8. Translations

All ICFs and Assents will be developed in English and translated into isiXhosa and Afrikaans based on the composition of the local population groups. Translation of the documents will

be outsourced to an accredited translation service. The study team will review the translations for consistency and to ensure the original meaning is retained through translation. The study team will pilot the translated documents either within the community or by review done by study team members, prior to use. Upon verification of the translation, the study PI/IoR designee will confirm that the document has been reviewed and that the translation is accurate. The translated documents need to be submitted to HREC and SAHPRA (if required). The documents need to be acknowledged by HREC. Proof of delivery and receipt at the SAHPRA offices will suffice as acknowledgment by the SAHPRA.

#### 151. **ABBREVIATIONS**

DAIDS	Division of AIDS
PI	Principal Investigator
IoR	Investigator of Record
SI	Sub Investigator
SOP	Standard Operating Procedure
IC	Informed Consent
ICC	Informed Consent Checklist
ICF	Informed Consent Form
ICH	International Conference on Harmonization
HREC-SU	Health Research Ethics Committee of Stellenbosch University
SAHPRA	South African Health Products Regulatory Authority (changed from MCC in February 2018)
QC	Quality Control

#### 152. **REFERENCES**

<b>Document</b>	<b>Current Version</b>
University of Stellenbosch Ethics Committee SOP	V4.3 dated June 2016
Department of Health, Ethics in Health Research	2015
SA GCP	Second edition 2016
Protection of Personal Information Act	

ICH Guidelines for Good Clinical Practice & Declaration of Helsinki	4 <sup>th</sup> version dated 9 November 2016
DAIDS SOP's <ul style="list-style-type: none"> <li>• Enrolling Children (including Adolescents) in Clinical Research: Clinical Research Site Requirements</li> <li>• Requirements for Informed Consent Development</li> <li>• Requirements for Source Documentation in DAIDS Funded and/or Sponsored Clinical Trials</li> </ul>	<ul style="list-style-type: none"> <li>• DWD-POL-CL-007.01; Effective date 24 Jul 09</li> <li>• DWD-POL-CL-02.00; Effective date 5 Feb 07</li> <li>• DWD-POL-CL-04.00; Effective date 5 Feb 07</li> </ul>

**153. REVIEW AND REVISION**

This document will be reviewed every 2<sup>nd</sup> year or earlier as required by the PI or designee.

**154. DOCUMENT REQUIREMENTS**

Study specific ICF and/or Assent

Informed Consent Checklist

**155. APPENDICES**

Appendix 1 – Informed Consent Checklist (may be made study specific)

**APPENDIX 1 – INFORMED CONSENT NOTES**

Participant/Parent/Legal Guardian Name: \_\_\_\_\_

\_\_\_\_\_

Screening ID: \_\_\_\_\_

Study: \_\_\_\_\_

**Informed Consent Notes**

<b>Element explained to the person giving consent</b>	<b>Yes</b>	<b>N/A</b>
<b>TIME STARTED</b>		
1. Participant/Parent/Legal Guardian has been asked to participate in <b>research</b> .		
2. The <b>purpose</b> of the research and <b>where</b> it will be conducted.		
3. The expected duration of the participant's involvement in the research.		
4. The total number of participants that will be involved at this site and/or South Africa and worldwide.		
5. A description of <b>all the processes and procedures</b> to which the participant will be subjected, emphasising any experimental procedures that may not have been used in routine medical practice.		
6. The <b>principal investigator's name and contact details</b> .		
7. Explanation of the <b>participant's responsibilities</b> .		
8. Explanation of the <b>randomization process</b> , the different treatment arms and the use of placebos (if applicable).		
9. Circumstances that may result in the <b>trial being terminated</b> or the <b>participant being withdrawn</b> .		
10. A description of foreseeable <b>risks and discomforts</b> .		
11. A description of potential <b>benefits to the participant or others</b> both during and after the research. If there are no expected direct benefits, the participant must specifically be made aware of this.		
12. Disclosure of <b>alternative procedures and course of treatments</b> available.		
13. Description of extent to which <b>confidentiality</b> will be maintained and protected.		
14. Statement that <b>sponsors of the study, study monitors or auditors or HREC members</b> may need to <b>inspect research records</b> .		

15. Statement that the <b>HREC has approved the research.</b>		
16. Contact details of the <b>HREC committee.</b>		
17. Explanation of how <b>research related injury</b> will be managed; ABPI compensation guidelines or other approved guidelines for serious research related injuries.		
18. Explanation as to <b>whom to contact in the event of research related injury.</b>		
19. Participation in the study is entirely <b>voluntary.</b>		
20. Participation is voluntary and participants are <b>free to withdraw</b> at any point without explanation or any negative consequences. Their routine health care will not be adversely affected.		
21. Participants must be informed of their rights to be told <b>any new relevant information</b> that arises during the course of the trial.		
22. That the study will be conducted according to <b>the International Declaration of Helsinki</b> and other applicable <b>international ethical codes</b> for research on human subjects.		
23. Explanation regarding <b>payment</b> for participation or <b>travel expenses.</b>		
24. Identity of the <b>sponsor</b> and any potential conflict of interests.		
25. Where appropriate, the participant should also be requested/advised to <b>inform his general practitioner</b> and life insurance company or medical aid of his/her participation.		
26. <b>Simple, clear language</b> has been used and all medical and technical terms have been explained.		
27. Where relevant, <b>the use of samples and storage for future</b> use has been addressed.		
The Informed Consent discussion was conducted as per <b>SOP GC001</b>	Yes	No
Participant/Parent/Legal Guardian has been given enough <b>time to ask questions</b>	Yes	No
Home Language of Participant:		
Language in which consent was obtained:		
	<b>TIME ENDED:</b>	
<b>A copy of the Informed Consent has been given to the participant/parent/legal guardian</b>	Yes	No

<p><b>Acknowledgment of receipt of consent form</b></p> <p>Name Participant/Parent/Legal Guardian: .....</p> <p>Signature Participant/Parent/Legal Guardian .....</p> <p>Date .....</p>
<p><b>Person taking consent</b></p> <p>Name: .....</p> <p>Signature .....</p> <p>Date .....</p>
<p><b>Comments</b></p> <p>.....</p> <p>.....</p> <p>.....</p> <p>.....</p> <p>.....</p> <p>.....</p> <p>.....</p> <p>.....</p>



## **B.13 Standard Operating Procedure 13: Detection of *M. tuberculosis* in pulmonary samples by smear microscopy, MGIT and GeneXpert MTB/RIF**

### **156. PURPOSE**

The purpose of this document is to describe the detection of *Mycobacterium tuberculosis* from pulmonary samples taken from children suspected to have pulmonary tuberculosis. Pulmonary samples: Gastric Aspirate (GA), Induced Sputum (IS) and Nasopharyngeal Aspirate (NPA) are analysed by using Smear Microscopy, MGIT liquid culture and GeneXpert real time PCR. Collected specimens are prepared first for all 3 methods of detection identically and are then split into 3 fractions to conduct the specific procedures.

### **157. INTRODUCTION AND PRINCIPLE**

Pulmonary samples require a digestion (liquefaction), decontamination, and concentration step in order to maximize the mycobacterial yield. The recommended procedure of decontamination to be used with the automated culture system (BD BACTEC MGIT 960) is the sodium hydroxide-N-Acetyl-L-Cysteine (NaOH-NALC) method followed by neutralization and concentration. The received pellet is further processed for 3 methods of detection: Smear Microscopy, MGIT liquid culture and GeneXpert real time PCR.

MGIT tubes containing liquid medium are supplemented with OADC and PANTA to promote mycobacterial growth and to inhibit non-mycobacterial growth. The processed and concentrated samples are inoculated into these supplemented MGIT tubes which are loaded into the instrument. The MGIT instrument continuously monitors the tubes for an increase in fluorescence and it flags tubes positive as soon as the detected fluorescence reaches a threshold. The time between start of incubation and detection (time to positivity, TTP) indicates the baseline bacterial load and the growth rate. The TTP correlates directly with the cycle threshold of the same sample determined by the GeneXpert instrument. The GeneXpert instrument detects the *rpoB* gene from the *M. tuberculosis* complex by using real time PCR and 5 fluorescent DNA probes. Samples for the measurements are mixed and inactivated with provided sample reagent and put into Xpert MTB/RIF cartridges. These cartridges are loaded into the instrument, processed and analysed by the software automatically.

**158. SPECIMENS**

Day 1 and Scheduled Visit (SV) Pulmonary samples (GA, IS, NPA) are collected from paediatric source patients after a recommended fast (nil-by-mouth) period into separate sterile containers. The minimal volume is 0.5 ml and rarely exceeds 5 ml. Specimens are processed at the NHLS Tygerberg laboratory.

**159. EQUIPMENT AND MATERIAL**

1. Disposable and sterile 50 ml plastic tubes.
2. Sterile NaOH-NALC-citrate solution in 1 l Scott glass bottle
3. Sterile sodium bicarbonate (NaHCO<sub>3</sub>) solution (100 g/l)
4. Phosphate buffer (PB, pH 6.8, 0.037 M)
5. Refrigerated centrifuge with a minimum of 3000-3500 x g force
6. Dispenser
7. Vortex mixer
8. Waste bag
9. Plastic Pasteur pipettes
10. 1 ml Gilson pipette and sterile pipette tips
11. Timer
12. BACTEC MGIT 960 instrument
13. MGIT tubes
14. MGIT growth supplement OADC
15. MGIT PANTA
16. Microscopy slides
17. Hot plate
18. Auramine-O stain
19. 0.5 % acid alcohol
20. Potassium permanganate solution
21. Carbol fuchsin
22. 3 % acid alcohol
23. Methylene blue
24. Distilled water
25. Bunsen burner
26. Microscope
27. Blood agar plate

28. Xpert MTB/RIF cartridges
29. Xpert sample reagent
30. GeneXpert instrument connected to a PC with GeneXpert Dx software

160. **SAMPLE RECEPTION**

1. Samples are collected from the patients by a study nurse and are accompanied by a completed sample request document.
2. The samples plus sample request documents are received on the 9<sup>th</sup> floor of Tygerberg Hospital at the NHLS sample reception office.
3. If samples are not processed immediately, store at 4-8 °C in the refrigerator for a maximum duration of 72 h.
4. Samples are logged into the NHLS laboratory information system (LIS) by a technician/receptionist.
5. The patient's name, surname, hospital number and sample type are linked to two unique sample numbers (TB### ### and STY### ###).
6. After registration the samples are taken to the NHLS TB Lab for processing.

161. **NAOH/SODIUM CITRATE-NALC DECONTAMINATION AND CONCENTRATION**

1. Decant a minimum of 3 ml and maximum of 10 ml sample into the labelled 50 ml centrifuge tube. For samples less than 3 ml; add PBS to a total volume of 3 ml (visually estimated using a pre-labelled 50 ml conical centrifuge tube for volumes less than the calibrated 5 ml). For samples greater than 10 ml
2. Start timer on 17 min and add an equal volume of NaOH-NALC solution to each specimen. Vortex lightly or hand mix for 15 sec.
3. Remove dispenser from NaOH-NALC solution and clean it with 70% alcohol.
4. Place the dispenser on the bottle with PBS.
5. Shake again for 1-2 min (do not exceed 20 minutes of decontamination).
6. Add PBS up to the 40 ml mark on the centrifuge tube to neutralize and mix by inverting.
7. Place the centrifuge tube and necessary counter balance into the centrifuge, close the lid, and centrifuge the samples for 20 min at 3000 x g at 4 °C.
8. Remove the tube after the centrifuge has stopped and decant supernatant completely.
9. Add 1.5 ml of PBS to the concentrated pellet and vortex to reconstitute.

10. Use the reconstituted pellet for Smear Microscopy, MGIT inoculation and GeneXpert processing (see below).

**162. PREPARATION OF SMEAR MICROSCOPY SLIDES**

1. Place 1 drop of TB precipitating fluid/fixative on a slide.
2. Add 1 drop of concentrated pellet ( $\pm 30 \mu\text{l}$ ) and mix using pipette tip.
3. The required smear size is between  $1.5 \times 1.5 \text{ cm}$  and  $2.0 \times 2.0 \text{ cm}$ .
4. Place slide on a hot tray for at least 2 hours at  $70^\circ\text{C}$  before staining.

**163. AURAMINE STAIN AND MICROSCOPY**

1. Fix slide with methanol for 30 sec.
2. Flood slide with Auramine-O stain and let it stand at room temperature for  $\pm 20 \text{ min}$ .
3. Rinse with tap water and drain.
4. Decolourise with 0.5% acid alcohol for  $\pm 2 \text{ minutes}$ .
5. Rinse with tap water and drain.
6. Flood smear with potassium permanganate solution and counter stain for  $\pm 2 \text{ min}$ .
7. Rinse with tap water and drain.
8. Let it air dry - Do not blot.
9. Analyze with a fluorescence microscope (using the 40X objective lens).
10. View stained slides under a fluorescent microscope and record results according to WHO/IUTLD standardized guidelines
11. Slides are read with the 40x objective microscope lens
12. Acid-fast bacilli emit a bright yellow fluorescence whilst cellular debris will be pale yellow in colour.

**164. INOCULATION AND INCUBATION OF MGIT TUBES**

1. Prepare one labelled MGIT tube for each specimen by adding 0.8 ml of reconstituted OADC/PANTA mixture using the 1 ml Gilson pipette.
2. Add 0.5 ml of reconstituted sample pellet to each prepared MGIT tube and mix by inverting. Place the tube into the BACTEC MGIT 960 instrument for maximum 42 days.
3. Positive and Negative MGIT tube results are automatically linked to the NHLS DISA system
4. Remove positive or negative flagged tubes from MGIT instrument.

5. Make a printout of the positive vials on the MGIT instrument; the time to positivity (TTP) is automatically linked to the sample tube on the NHLS DISA system.

**165. BACTEC MGIT 960 SYSTEM (CULTURE)**

1. The BACTEC system will record MGIT tubes with NO growth (No change in tube fluorescence) after 42 days as NEGATIVE.
2. The BACTEC system will record MGIT tubes with sufficient organism growth (significant change in tube fluorescence) as POSITIVE and will provide a time to positivity (TTP).
3. NEGATIVE and POSITIVE culture reports will be printed separately on the BACTEC system.

**166. CONTAMINATION TESTING OF MGIT CULTURES**

1. Divide a blood agar plate into squares and label them with sample IDs of positive tubes.
2. Label clean microscopy slides with sample IDs of positive MGIT tubes and place one drop of fixing solution on each slide. Do not let it dry.
3. Use a sterile plastic pipette to remove some of the growth from the bottom of the MGIT vial. NB: Do not remove all the growth.
4. Inoculate one blood agar square with one drop of suspension and let it dry in the biosafety cabinet. Emulsify the rest of the suspension on the labeled microscopy slide.
5. Allow smears to dry on the hot plate for at least 2 hours at 80°C.
6. Place the blood agar plate in the incubator for 2 days at 35 °C, but check the plate every day.
7. Do ZN staining (see below) on the microscopy slide.

### 167. PROCESSING OF POSITIVE CULTURES

Every MGIT POSITIVE tube has a ZN smear and TB Ag MPt64 antigen test done. Table 13 explains culture positive result interpretation and further testing

**Table 10.13. Interpretation of culture positive specimen results**

MGIT	ZN	TB Ag MPt64	Further Processing	HAIN MTBDR Test	HAIN CM	Final Result Reporting
+	+ (Cording)	+/-	NONE	YES	Only if MTBDR negative	POSITIVE and TTP OR NTM
+	+ (Not Cords)	+/-	NTM testing	NO*	YES	NTM OR POSITIVE and TTP
+	-	+	NONE	YES	NO	POSITIVE and TTP OR CONTAMINATED and TTP
+	-	-	NONE	NO	NO	CONTAMINATED and TTP
-	N/A	NONE	IF VISIBLE GROWTH	NONE	NONE	NONE

### 168. ZIEHL-NEELSEN STAINING

1. Flood the slide with carbol fuchsin.
2. Gently heat the slide with Bunsen burner until steam rises for 1 min. Do not boil.
3. After 4 -5 min wash off carbol fuchsin with water
4. Flood the slide with 3% acid alcohol and wait for 2 min. Decolourise until no more colour drains from the slide.
5. Rinse gently with running water.
6. Flood the slide with methylene blue for counter staining and wait 1 min.
7. Rinse with water and let air dry – Do not blot.
8. Analyse with light microscope (100 X objective lens).

**169. RAPID TB AG MPT64 ANTIGEN TEST (BIOLINE)**

1. Processed according to package insert
2. Remove required amount of devices from the foil pouch and number it.
3. Working inside the biosafety cabinet, Add 100µl of liquid culture with a pipette into the sample well.
4. As the test begins to work, a purple colour moves across the result window in the centre of the test device
5. Interpret the test result 15 minutes after sample application

**170. HAIN MTBDR TEST REPORTING**

1. Processed as per the manufacturer's instructions (HAIN GenoLyse VER 1.0, Hain Lifescience GmbH, Nehren, Germany) using 1 ml of positive MGIT culture.
2. Results reported as in Table 14 below

**Table 10.14. HAIN MTBDR reporting**

<b>HAIN MTBDR Results</b>						
<b>MTBC</b>	<b>RIF</b>	<b>INH</b>	<b>2nd line tests</b>	<b>NTM ID</b>	<b>LIS Reporting 1st line</b>	<b>LIS Reporting 2nd line</b>
Negative	N/A	N/A	N/A	YES	NTM - species	N/A
Positive	-	-	NONE	NO	MTB POS; RIF sensitive; INH sensitive	N/A
Positive	+	-	YES	NO	MTB POS; RIF resistant; INH sensitive	Ofloxacin and Amikacin
Positive	-	+	YES	NO	MTB POS; RIF sensitive; INH resistant	Ofloxacin and Amikacin
Positive	+	+	YES	NO	MTB POS; RIF resistant; INH resistant	Ofloxacin and Amikacin

**171. HAIN COMMON MYCOBACTERIA (CM)**

1. Processed as per the manufacturer's instructions (GenoType Mycobacterium CM VER 1.0, Hain Lifescience GmbH, Nehren, Germany) using 1 ml of positive MGIT culture.
2. Results reported as Mycobacterial species if not identified.

**172. RESULT REPORTING AND INTERPRETATION**

All lab results (smear, culture, Xpert and DST) are recorded in the NHLS laboratory information system (LIS).

173.

**174. SAMPLE PREPARATION FOR GENEXPERT**

1. Prepare sample reagent, cartridge, waste bin and bag, 3 ml plastic Pasteur pipettes, and the reconstituted sample pellet.
2. Label 50 ml centrifuge tube and cartridge with sample ID sticker on lid.
3. Pipette 1.0 ml of the reconstituted sample pellet into the prepared and labelled 50 ml centrifuge tube.
4. Add 2.0 ml of Xpert MTB/RIF sample reagent to the sample in the 50 ml centrifuge tube (for a dilution of 1:2) and invert 10 times (Don't vortex!). Note: If less than 0.7 ml sample sediment is left over use a dilution ratio of 1:3 to ensure that the sample + Xpert sample reagent total volume is  $\geq 2$  ml (Minimum required volume).
5. Incubate for 15 min at room temperature and invert again 10 times after 8 min incubation.
6. Open the lid of the cartridge, check the filter and for any leakages.
7. Transfer the 2.0-3.0 ml mixture completely into the corresponding labelled cartridge, close the cartridge lid, wipe the cartridge with Incidin, and take it to the GeneXpert machine.
8. The cartridge must be used within 8 hours after opening.
9. Store the remaining sample ( $\pm 0.5$  ml) in the refrigerator at 4 °C in case the measurement has to be retested. Repeat Xpert test once if Error/Invalid/No result occurs.

**175. GENEXPERT MEASUREMENT**

1. Switch on GeneXpert machine and PC, start the GeneXpert Dx software and log in.
2. Click on "Create test" to start the measurement.



3. Scan the sample barcode followed by the cartridge barcode, press “start” and enter your password.
4. Load the cartridge into the indicated module (flashing light) and close the module gate.
5. Each measurement requires 1 hour 55 minutes and is conducted automatically.
6. After the measurement is finalized the result is automatically linked to the NHLS DISA system and results are archived on the GeneXpert PC.
7. Optional: Open the pdf-file by clicking on “Preview pdf” and print the result page.
8. Close the software, shut down the PC, and switch off the instrument.

#### 176. **POSSIBLE PROBLEMS**

In case the GeneXpert instrument reports an error or an indeterminate result: Fetch the stored pellet specimen and repeat sample preparation and measurement as above.

In case the cartridge is dropped or falls over and a spillage occurs: Clean the spillage, discard the cartridge, fetch the stored pellet specimen, and repeat sample preparation and measurement as above.

#### 177. **QUALITY CONTROL**

**Positive control:** H37Rv (once a week)

**Negative control:** 5 ml PBS (once a day)

Process negative and positive controls with the samples; put both controls at the end of the batch: first positive followed by negative control.

Use the same decontamination/inoculation/microscopy protocol as above.

#### 178. **REPORTING**

The MGIT result is reported as positive, negative, or contaminated determined by microscopy and blood agar. A positive result must contain the TTP reported by the MGIT instrument.

The GeneXpert measurement is reported as MTB detected, MTB not detected, or invalid/error. RIF resistance is either “detected”, “not detected” or “indeterminate”. A MTB positive result must contain the cycle threshold ( $C_T$ ) of probe B according to published data. Detected resistances against rifampicin have to be reported to the sites.

#### 179. **REVIEW AND REVISION**

This SOP was in use from March 2013 until June 2014.

## **B.14 Standard Operating Procedure 14: Detection of *Mycobacterium tuberculosis* in pooled pulmonary samples by smear microscopy, MGIT and GeneXpert MTB/RIF**

### **180. PURPOSE**

The purpose of this document is to describe the detection of *Mycobacterium tuberculosis* in gastric aspirates (GA), nasopharyngeal aspirates (NPA) and induced sputa (IS) samples taken from children suspected to have pulmonary tuberculosis by using MGIT liquid culture, smear microscopy and a GeneXpert (GXP) real time PCR test. A total of 6 Specimens will be collected from each patient over a 2 day period: 3 samples (1 x GA, 1 x IS and 1 x NPA) on day 1 and 3 samples (1 x GA, 1 x IS and 1 x NPA) on day 2. Collected specimens are decontaminated to eliminate most bacterial species leaving only viable TB bacilli for culture.

### **181. INTRODUCTION AND PRINCIPLE**

GA, NPA and IS require a digestion (liquefaction), decontamination, and concentration step in order to maximize the mycobacterial yield. The recommended procedure of decontamination will be used on each sample type with the automated culture system BACTEC MGIT 960 and the sodium hydroxide-N-Acetyl-L-Cysteine (NaOH-NALC) method followed by neutralization and concentration. The received samples on Day 1 and Day 2 will be processed by 2 separate methods of detection:

Day 1 samples (GA, NPA, IS) will have a separate individual MGIT liquid culture, Auramine stain and a GeneXpert real time PCR for each sample type.

Day 2 samples (GA, NPA, IS) will have 1 combined (pooled) MGIT liquid culture, 1 combined (pooled) Auramine stain and 1 combined (pooled) GeneXpert real time PCR for all 3 sample types together (GA + NPA + IS).

MGIT tubes containing liquid medium are supplemented with OADC (enrichment media) and PANTA (antibiotic mixture) to promote mycobacterial growth and to inhibit non-mycobacterial growth. The processed and concentrated samples are inoculated into these supplemented MGIT tubes which are loaded into the automated culture system. The MGIT instrument continuously monitors the tubes for an increase in fluorescence and it flags tubes positive as soon as the detected fluorescence reaches a threshold. The time between

start of incubation and detection (time to positivity, TTP) indicates the baseline bacterial load and the growth rate. The TTP correlates directly with the cycle threshold of the same sample determined by the GeneXpert instrument. The GeneXpert instrument detects the *rpoB* gene from the *M. tuberculosis* complex by using real time PCR and 5 fluorescent DNA probes. All GeneXpert samples are inactivated with provided sample reagent and placed into Xpert MTB/RIF cartridges. These cartridges are loaded into the instrument, processed and analysed by the software automatically.

## 182. SPECIMENS

Day 1 and Day 2 samples: Early morning gastric aspirate, nasopharyngeal aspirate and induced sputum samples are collected from paediatric source patients and are placed in separate sterile containers. The minimal volume is 0.5 ml and rarely exceeds 5 ml.

Specimens are processed at the NHLS Tygerberg laboratory.

Day 1 samples (GA, NPA, IS) are placed in separate sample bags along with the accompanied sample request document of each.

Day 2 samples (GA, NPA, IS) from the same patient are placed into one sample bag together with 1 sample request document for transport to the lab.

## 183. EQUIPMENT AND MATERIAL

1. Disposable and sterile 50 ml plastic tubes.
2. Sterile NaOH-NALC-citrate solution in 1 l Scott glass bottle
3. Sterile sodium bicarbonate ( $\text{NaHCO}_3$ ) solution (100 g/l)
4. Phosphate buffer (PB, pH 6.8, 0.037 M)
5. Refrigerated centrifuge with a minimum of 3000-3500 x g force
6. Dispenser
7. Vortex mixer
8. Waste bag
9. Plastic Pasteur pipettes
10. 1 ml Gilson pipette and sterile pipette tips
11. Timer
12. BACTEC MGIT 960 instrument
13. MGIT tubes

14. MGIT growth supplement ODAC
15. MGIT PANTA
16. Microscopy slides
17. Hot plate
18. Auramine-O stain
19. 0.5 % acid alcohol
20. Potassium permanganate solution
21. Carbol fuchsin
22. 3 % acid alcohol
23. Methylene blue
24. Distilled water
25. Bunsen burner
26. Microscope
27. Blood agar plate
28. Xpert MTB/RIF cartridges
29. Xpert sample reagent
30. GeneXpert instrument connected to a PC with GeneXpert Dx software

184. **SAMPLE RECEPTION**

1. Samples are collected from the patients by a study nurse and are accompanied by a completed sample request document (SAM1/REQ1 form).
2. The samples plus sample request documents are received on the 9th floor of Tygerberg Hospital at the NHLS sample reception office.
3. If samples are not processed immediately, store at 4-8 °C in the refrigerator for a maximum duration of 72 h.
4. Samples are logged into the NHLS laboratory information system (LIS) by a technician/receptionist.
5. The patient's name, surname, hospital number and sample type are linked to two unique sample numbers (TB### ### and STY### ###).
6. After registration the samples are taken to the NHLS TB Lab for processing.
7. Day 1 samples are processed according to "SOP detection of MTB in pulmonary samples V1.0"

8. Day 2 (Pooled) samples are processed according to this SOP (“SOP detection of MTB in pooling V1.0”)

#### 185. **NAOH/SODIUM CITRATE-NALC DECONTAMINATION AND CONCENTRATION**

1. Decant a maximum of 10 ml sample into the labelled 50 ml centrifuge tube.
2. Start timer on 18 min and add an equal volume of NaOH-NALC solution to each specimen. Vortex lightly or hand mix for 15 sec.
3. Shake again for 1-2 min.
4. Remove dispenser from NaOH-NALC solution and clean it with 70% alcohol.
5. Place the dispenser on the bottle with PBS (Phosphate Buffer).
6. Incubate the specimen/NaOH-NALC mix for a maximum duration of 18 min.
7. Add PBS up to the 40 ml mark on the centrifuge tube to neutralize and mix by inverting.
8. Place the centrifuge tube and necessary counter balance into the centrifuge, close the lid, and centrifuge the samples for 20 min each at 3000 x g at 4 °C.
9. Remove the tube after the centrifuge has stopped and decant supernatant completely.
10. For Day 2 Samples add 1 ml phosphate buffer to each tube (GA, IS, NPA) and add all 3 fractions into the GA tube. (This tube will now be the Pooled specimen tube i.e will contain the reconstituted pellets of all 3 sample types GA + IS + NPA; ± 3 ml)
11. Use the reconstituted pellet from the Pooled specimen tube for Smear Microscopy, MGIT inoculation and GeneXpert processing (see below).

#### 186. **PREPARATION OF MICROSCOPY SLIDES**

1. Place 1 drop of TB precipitating fluid/fixative on a slide.
2. Add 1 drop of reconstituted pooled sample pellet (± 30 µl) and mix using pipette tip.
3. The required smear size is between 1.5 x 1.5 cm and 2.0 x 2.0 cm.
4. Place slide on a hot tray for at least 2 hours at 70°C before staining.

#### 187. **AURAMINE STAIN AND MICROSCOPY**

1. Fix slide with methanol for 30 sec.
2. Flood slide with Auramine-O stain and let it stand at room temperature for ± 20 min.
3. Rinse with tap water and drain.
4. Decolourise with 0.5% acid alcohol for ± 2 minutes.

5. Rinse with tap water and drain.
6. Flood smear with potassium permanganate solution and counter stain for  $\pm$  2 min.
7. Rinse with tap water and drain.
8. Let it air dry - Do not blot.
9. Analyse with a fluorescence microscope (using the 40X objective lens).

#### **INOCULATION AND INCUBATION OF MGIT TUBES**

1. Prepare one labelled MGIT tube for each specimen by adding 0.8 ml of reconstituted OADC/PANTA using the 1 ml Gilson pipette.
2. Add 0.5 ml of reconstituted pooled sample pellet to one prepared MGIT tube and mix by inverting. Place the tube into the BACTEC MGIT 960 instrument for maximum 42 days.
3. Positive and Negative MGIT tube results are automatically linked to the NHLS DISA system
4. Remove positive or negative flagged tubes from MGIT instrument.
5. Make a printout of the positive vials on the MGIT instrument; the time to positivity (TTP) is automatically linked to the sample tube on the NHLS DISA system.

#### **188. CONTAMINATION TESTING OF MGIT CULTURES**

1. Divide a blood agar plate into squares and label them with sample IDs of positive tubes.
2. Label clean microscopy slides with sample IDs of positive MGIT tubes and place one drop of fixing solution on each slide. Do not let it dry.
3. Use a sterile plastic pipette to remove some of the growth from the bottom of the MGIT vial. NB: Do not remove all the growth.
4. Inoculate one blood agar square with one drop of suspension and let it dry in the biosafety cabinet. Emulsify the rest of the suspension on the labeled microscopy slide.
5. Allow smears to dry on the hot plate for at least 2 hours at 80°C.
6. Place the blood agar plate in the incubator for 2 days at 35°C, but check the plate every day.
7. Do ZN staining (see below) on the microscopy slide.

#### **189. ZIEHL-NEELSEN STAINING**

1. Flood the slide with carbol fuchsin.
2. Gently heat the slide with Bunsen burner until steam rises for 1 min. Do not boil.
3. After 4 -5 min wash off carbol fuchsin with water
4. Flood the slide with 3% acid alcohol and wait for 2 min. Decolourise until no more colour drains from the slide.
5. Rinse gently with running water.
6. Flood the slide with methylene blue for counter staining and wait 1 min.
7. Rinse with water and let air dry – Do not blot.
8. Analyse with light microscope (100 X objective lens).

#### 190. **SAMPLE PREPARATION FOR GENEXPERT**

1. Prepare sample reagent, cartridge, waste bin and bag, 3 ml plastic Pasteur pipettes, and the reconstituted sample pellet.
2. Label 50 ml centrifuge tube and cartridge with sample ID sticker on lid.
3. Pipette 1.0 ml of the reconstituted sample pellet into the prepared and labelled 50 ml centrifuge tube. Note: Remember to use the reconstituted “pooled” sample pellet for Day 2 samples.
4. Add 2.0 ml of Xpert MTB/RIF sample reagent to the sample in the 50 ml centrifuge tube (for a dilution of 1:2) and invert 20 times (Don't vortex!). Note: If less than 0.7 ml sample sediment is left over use a dilution ratio of 1:3 to ensure that the sample + Xpert sample reagent total volume is  $\geq 2$  ml (Minimum required volume).
5. Incubate for 15 min at room temperature and invert again 10 times after 8 min incubation.
6. Open the lid of the cartridge, check the filter and for any leakages.
7. Transfer the 2.0-3.0 ml mixture completely into the corresponding labelled cartridge, close the cartridge lid, wipe the cartridge with Incidin/ethanol, and take it to the GeneXpert machine.
8. The cartridge must be used within 8 hours after opening.
9. Store the remaining sample ( $\pm 0.5$  ml) in the refrigerator at 4 °C in case the measurement has to be retested. For longer storage ( $> 2$  days) freeze it away in a cryovial at -20 or -80 °C. Repeat Xpert test once if Error/Invalid/No result occurs.

**191. GENEXPERT MEASUREMENT**

1. Switch on GeneXpert machine and PC, start the GeneXpert Dx software and log in.
2. Click on “Create test” to start the measurement.
3. Scan the sample barcode followed by the cartridge barcode, press “start” and enter your password.
4. Load the cartridge into the indicated module (flashing light) and close the module gate.
5. Each measurement requires 1 hour 55 minutes and is conducted automatically.
6. After the measurement is finalized the result is automatically linked to the NHLS DISA system and results are archived on the GeneXpert PC.
7. Optional: Open the pdf-file by clicking on “Preview pdf” and print the result page.
8. Close the software, shut down the PC, and switch off the instrument.

**192. BACTEC MGIT 960 SYSTEM (CULTURE)**

1. The BACTEC system will record MGIT tubes with NO growth (No change in tube fluorescence) after 42 days as NEGATIVE.
2. The BACTEC system will record MGIT tubes with sufficient organism growth (significant change in tube fluorescence) as POSITIVE and will provide a time to positivity (TTP).
3. NEGATIVE and POSITIVE culture reports will be printed separately on the BACTEC system.

**193. PROCESSING OF POSITIVE CULTURES**

Every MGIT POSITIVE tube has a ZN smear and TB Ag MPt64 antigen test done. Table 15 explains culture positive result interpretation and further testing

**Table 10.15. Interpretation of culture positive specimen results**

<b>MGIT</b>	<b>ZN</b>	<b>TB Ag MPt64</b>	<b>Further Processing</b>	<b>HAIN MTBDR Test</b>	<b>HAIN CM</b>	<b>Final Result Reporting</b>
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+	+(Cording)	+/-	NONE	YES	Only if MTBDR negative	POSITIVE and TTP OR NTM
+	+(Not Cords)	+/-	NTM testing	NO*	YES	NTM OR POSITIVE and TTP
+	-	+	NONE	YES	NO	POSITIVE and TTP OR CONTAMINATED and TTP
+	-	-	NONE	NO	NO	CONTAMINATED and TTP
-	N/A	NONE	IF VISIBLE GROWTH	NONE	NONE	NONE

**194. RAPID TB AG MPT64 ANTIGEN TEST (BIOLINE)**

1. Processed according to package insert
2. Remove required amount of devices from the foil pouch and number it.
3. Working inside the biosafety cabinet, Add 100µl of liquid culture with a pipette into the sample well.
4. As the test begins to work, a purple colour moves across the result window in the centre of the test device
5. Interpret the test result 15 minutes after sample application

**195. HAIN MTBDR TEST REPORTING**

1. Processed as per the manufacturer's instructions (HAIN GenoLyse VER 1.0, Hain Lifescience GmbH, Nehren, Germany) using 1 ml of positive MGIT culture.
2. Results reported as in Table 16 below

**Table 10.16. HAIN MTBDR reporting**

<b>HAIN MTBDR Results</b>
---------------------------

MTBC	RIF	INH	2nd line tests	NTM ID	LIS Reporting 1st line	LIS Reporting 2nd line
Negative	N/A	N/A	N/A	YES	NTM - species	N/A
Positive	-	-	NONE	NO	MTB POS; RIF sensitive; INH sensitive	N/A
Positive	+	-	YES	NO	MTB POS; RIF resistant; INH sensitive	Ofloxacin and Amikacin
Positive	-	+	YES	NO	MTB POS; RIF sensitive; INH resistant	Ofloxacin and Amikacin
Positive	+	+	YES	NO	MTB POS; RIF resistant; INH resistant	Ofloxacin and Amikacin

#### 196. HAIN COMMON MYCOBACTERIA (CM)

1. Processed as per the manufacturer's instructions (GenoType Mycobacterium CM VER 1.0, Hain Lifescience GmbH, Nehren, Germany) using 1 ml of positive MGIT culture.
2. Results reported as Mycobacterial species if not identified.

#### 197. RESULT REPORTING AND INTERPRETATION

All lab results (smear, culture, Xpert and DST) are recorded in the NHLS laboratory information system (LIS).

#### 198. POSSIBLE PROBLEMS

In case the GeneXpert instrument reports an error or an indeterminate result: Fetch the stored pellet specimen and repeat sample preparation and measurement as above.

In case the cartridge is dropped or falls over and a spillage occurs: Clean the spillage, discard the cartridge, fetch the stored pellet specimen, and repeat sample preparation and measurement as above.

#### 199. QUALITY CONTROL

Positive control: H37Rv (once a week)

Negative control: 5 ml PB (once a day)

Process negative and positive controls with the samples; put both controls at the end of the batch: first positive followed by negative control.

Use the same decontamination/inoculation/microscopy protocol as above.

#### 200. **REPORTING**

The MGIT result is reported as positive, negative, or contaminated determined by microscopy and blood agar. A positive result must contain the TTP reported by the MGIT instrument.

The GeneXpert measurement is reported as MTB detected, MTB not detected, or invalid/error. RIF resistance is either “detected”, “not detected” or “indeterminate”. A MTB positive result must contain the cycle threshold (CT) of probe B according to published data. Detected resistances against rifampicin have to be reported to the sites.

#### 201. **REVIEW AND REVISION**

This SOP was in use from June 2014 until study end.

### **B.15 Standard Operating Procedure 15: Detection of**

### ***Mycobacterium tuberculosis* in stool samples by smear, culture and GeneXpert MTB/RIF**

#### 202. **BACKGROUND**

Stool specimens require a liquefaction, decontamination and concentration step in order to maximize the mycobacterial yield. The recommended procedure for decontamination is the sodium hydroxide-N-Acetyl-L-Cysteine (NaOH-NALC) method followed by neutralization and concentration used with the automated BACTEC MGIT 960 culture system. The concentrated pellet is further processed for 3 methods of detection: smear microscopy, MGIT liquid culture and GeneXpert (Xpert) MTB/RIF real time PCR.

MGIT bottles are supplemented with OADC and PANTA to promote mycobacterial growth and to limit growth of non-mycobacterial contaminating species. The decontaminated and concentrated specimens are inoculated into the supplemented MGIT tubes which are loaded into BACTEC MGIT 960 culture system. The BACTEC MGIT 960 culture system continuously monitors the MGIT bottles to detect an increase in fluorescence and it flags the bottle as positive when fluorescence levels reach a specific threshold. The time between incubation and detection (time to positivity, TTP) indicates the initial bacterial load and the growth rate. The TTP correlates directly with the cycle threshold of the same sample determined by the Xpert instrument (1).

The Xpert MTB/RIF instrument detects the *rpoB* gene from the *M. tuberculosis* complex by using real time PCR and 5 fluorescent DNA probes. Specimens for measurements are mixed and inactivated with provided sample reagent and pipetted into Xpert MTB/RIF cartridges. These cartridges are loaded into the instrument, processed automatically and analysed by the software (2).

### 203. OBJECTIVES

The purpose of this document is to describe the detection of *Mycobacterium tuberculosis* in stool samples from children suspected to have pulmonary tuberculosis by smear microscopy, MGIT liquid culture and Xpert real time PCR. Collected specimens are prepared first for methods of detection identically and are then split in three fractions to conduct the specific procedures.

### 204. EQUIPMENT AND MATERIAL

At NHLS TB Laboratory:

1. Disposable and sterile 50 ml plastic tubes (Falcon Tube).
2. Phosphate buffer (PBS, pH 6.8, 0.037 M)
3. Refrigerated centrifuge with a minimum of 3000 x g (Centrifugal force)
4. Vortex mixer
5. Waste bag
6. Plastic Pasteur pipettes
7. Timer
8. Laboratory Coat

9. Disposable gloves
10. 70% Alcohol (or Ethanol or EtOH)
11. Biosafety Cabinet Class II
12. Specimen rack
13. Marker
14. N95 Mask
15. Scale (Adam AFP-1200L, max 1200g, smallest division = 10mg)
16. Xpert MTB/RIF cartridges
17. Xpert sample reagent
18. Glass microscope slides ( )
19. Slide Fixative Solution
20. Heating Block
21. Light microscope
22. Fluorescent microscope
23. Timer
24. BBL BACTEC MGIT 960 system
25. BBL PANTA (Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprin, Azlocillin)
26. BBL OADC (Oleic acid, Albumin, Dextrose, Catalase)
27. BBL MGIT tubes (Mycobacterium Growth Indicator Tube)
28. Staining material (ZN and Auramine)
29. Microscope immersion oil
30. Dark room

At Task Laboratory Fisan building:

1. GeneXpert 4 module instrument connected to a PC with GeneXpert Dx software (GeneXpert Dx software version 4.4a)
2. Printer

## 205. SAFETY PRECAUTIONS

Standard safety operating procedures are to be followed at all times and all material should be treated as potentially infectious.

Before and after the specimens have been processed the laboratory work surface should be disinfected with 70% Alcohol.

All stool specimens are processed within the Biosafety Cabinet using a N95 mask.

## 206. HANDLING OF SPECIMEN IN THE CLINIC

1. Collect a fresh stool specimen from a paediatric source patient (study participant) into a sterile container as per stool collection SOP. The minimal volume is 0.3 grams (g) and should not exceed 5g. The specimen should be collected in a 25ml Faecal Cup with spoon container.
2. Label the container and complete the appropriate forms
3. Place the specimen and accompanied documentation (SAM1 form) in a transport bag
4. The specimen must be transported at 2-8°C and kept in the lab fridge (DTTC Room 0073).
5. A stool specimen is taken at each visit
6. Visits and specimens are given unique lab codes (*Table 17*)

**Table 10.17. Stool investigation schedule**

Stool Specimens				
Visit	Investigations			
Test Type	Auramine stain	Liquid culture (MGIT)	GeneXpert (Decon Specimen)	Storage
<b>Baseline</b>	X	X	X	X
<b>Month 1</b>	X	X	X	X
<b>Month 2</b>	X	X	X	X
<b>Month 4</b>	X	X	X	X
<b>Month 6</b>	X	X	X	X
<b>Unscheduled</b>	X	X	X	X

**Table 10.18. Treatment Response Lab Codes**

<b>Treatment Response Lab Codes</b>
-------------------------------------

Visits		Specimen Types	
BL	Baseline	A/B	Gastric Aspirate/Expectorated Sputum
M1	Month 1	C/D	Induced Sputum
M2	Month 2	E	Stool
M4	Month 4	F/G	Naso Gastric String
M6	Month 6	H/I	Nasopharyngeal Aspirate
UNS	Unscheduled Visit	P	Pooled specimen
SV	Scheduled Visit		

#### 207. SPECIMEN RECEPTION BY NURSING AND DATA TEAM

1. The nurse brings the specimens collected in the ward to DTTC room 0073 in their transport boxes (with ice bricks)
2. All sample barcodes are checked in accordance to the sample transport form (SAM1)
3. All forms are captured and the Treatment Response sample log file is completed
4. Specimens are placed in refrigerator 2-8°C

#### 208. SPECIMEN RECEPTION BY LABORATORY TEAM (FOR LAB PERSONNEL ONLY)

1. The laboratory technician signs the Treatment Response sample log sheet (*Figure 10*).
2. Specimens are collected and transported in a cold box to the NHLS lab on the 9<sup>th</sup> floor of Tygerberg Hospital (room 322) for processing.
3. Working inside the Biosafety Cabinet, remove specimens from bag and check that the specimens and barcodes match according to the SAM1 – DTTC Sample Form that accompanies each specimen.

TREATMENT RESPONSE SAMPLE LOG											
DATE	SUBJECT BARCODE	VISIT	SAMPL E TYPE	SAMPLE BARCODE	SIGNED IN BY		RECEIVED BY		LOGGED	RECEIVED IN LAB	
					RA CODE	SIGNATURE	RA CODE	SIGNATURE		RA CODE	SIGNATURE
YYYY-MM-DD	6299900001	BL	STL	0044####	001		002		YES	003	

**Figure 10.10. Example of Treatment Response sample log**

#### 209. CONTAINER PREPARATION AND LAB LOG BOOK

1. Label 50ml centrifuge tubes, glass slides, and MGIT tubes with the corresponding Subject barcode (PID) and unique lab specimen barcode (-80°C barcode).

2. Enter Subject barcode (PID) and specimen details (*Figure 11*) into the lab log book (Results only entered once completed).
3. No clinical data should be attached to the specimen; information about the patient must be anonymous.
4. If samples are not processed immediately, store at 2-8 °C in the refrigerator for a maximum duration of 72 h.

LAB NO	DATE	SUBJECT BARCODE	SOURCE	AURAMINE	MGIT	GXP			ZN	STORAGE	COMMENTS
1A	YYYY-MM-DD	62299900001	STL	00521000	00521001	00521002			00521003	00521004	TB# : 712 ### STY# : 460 ####
				NEGATIVE	POSITIVE; 10D	POS	LOW	S	POSITIVE	00521005	Sample# : 00521006

**Figure 10.11. Lab log book example**

## 210. REAGENT PREPARATION

1. Prepare sodium hydroxide – N-Acetyl-L-Cysteine (NaOH-Nalc)
  - a. Prepare fresh daily
  - b. Calculate the volume that will be required to process the days' specimens (approx. 5ml per specimen).
  - c. Add equal volumes of NaOH (5% initial concentration) and sodium (NA) citrate (2.9%)
  - d. Weigh out, and add, N-acetyl-L-cystein (Nalc) as required; 0.5g per 100ml NaOH-Na citrate mixture
  - e. Shake by hand to dissolve Nalc powder
  - f. Final NaOH concentration will be 1.25% when added to the specimens
  
2. Prepare BD MGIT tubes
  - a. This mixture can be kept refrigerated (2-8°C) for up to 2 days
  - b. Fetch (PANTA)/(OADC) from reagent fridge
  - c. Reconstitute lyophilised PANTA with 15ml OADC supplement (as per manufacturer's instructions (1))
  - d. Add 0.8 ml of reconstituted PANTA/OADC mixture to each pre-labelled MGIT tube using the 1 ml Gilson pipette



**211. DECONTAMINATION**

1. The below steps are performed under a Biosafety Cabinet at NHLS in room 322 wearing a N95 mask.
2. Only open one lid at a time. Patient specimens must be processed independently to avoid a mix up.
3. Add 5-10ml PBS buffer to the specimen(s) in the original 25ml Faecal Cup with spoon container (Do one specimen at a time and avoid cross contamination)
4. Close Faecal cup securely
5. Completely homogenise the specimen (in the PBS buffer) by Vortex mixing for approximately 20 seconds (or longer to ensure that specimen is thoroughly homogenised).
6. From the homogenised specimen(s) transfer 5ml of the stool/PBS buffer supernatant into the corresponding previously labelled 50ml centrifuge tube.
7. Store the remaining stool/PBS buffer specimen at -20°C (ensure that PID and sample barcode are still on the tube)
8. Add an equal volume of prepared NaOH-Nalc solution to each specimen. Start timer on 17 minutes. Vortex briefly or hand mix for 15 sec.
9. Incubate at room temperature (RT) inside safety cabinet until timer is done.
10. To each specimen, add PBS buffer up to the 40 ml mark on the 50ml centrifuge tube(s) to neutralize and mix by inverting 10 times.

**212. CONCENTRATION**

1. Ensure that centrifuge is set at 3000 x g, 4°C and 20min.
2. Place the 50ml centrifuge tube(s) and necessary counter balance into the centrifuge, close the lid, and press start to centrifuge the specimen(s).
3. After centrifugation, remove the 50ml Falcon tubes containing the specimens and, inside the Biosafety Cabinet, decant the supernatant completely in the liquid-waste bucket.
4. Using a 3ml sterile Pasteur pipette; add 2ml PBS buffer to the concentrated specimen pellet and mix by pipetting up and down until completely homogenised.
5. Inoculate 0.5ml of the resuspended pellet into the corresponding MGIT tube and incubate in the BACTEC MGIT 960 system

6. Place a drop (0.1ml) of the resuspended pellet on a glass slide containing fixative
7. Use 1ml of the remaining pellet for Xpert protocol

**213. BACTEC MGIT 960 SYSTEM (CULTURE)**

1. Touch the alarm soft touch button
2. Find drawer where the last specimens were added
3. Open the required drawer
4. Press soft touch button below “tube entry” icon
5. Scan the barcode located on the MGIT tube 1<sup>st</sup> and secondly scan the -80°C sample barcode also located on the MGIT.
6. Place the tube in the corresponding well (well with green light flashing)
7. Once all MGIT tubes have been inserted; close the drawer
8. Tubes are incubated for up to 42 days

**214. PREPARATION OF AURAMINE MICROSCOPY SLIDES**

1. Place 1 drop of TB precipitating fluid/fixative (NHLS fridge) on a slide.
2. Add 1 drop ( $\pm$  0.1ml) of the resuspended specimen pellet and mix.
3. The required smear size is between 1.5 x 1.5 cm and 2.0 x 2.0 cm.
4. Carefully place slide on a hot tray (60°C) and allow to dry for at least 2 hours (heat kill) before staining.

**215. AURAMINE O STAIN AND MICROSCOPY**

1. Working in the fume hood, fix prepared (heat inactivated) slide with methanol for 30 sec.
2. Flood slide with Auramine O stain and let it stand at RT for 20 min.
3. Rinse with tap water and drain.
4. Decolorize with 0.5% acid alcohol for 2 minutes.
5. Rinse with tap water and drain.
6. Flood smear with potassium permanganate solution (counter stain) for 2 min.
7. Rinse with tap water and drain.
8. Allow slides to air dry - Do not blot.

**216. SAMPLE PREPARATION FOR GENEXPERT**

1. Label an Xpert MTB/RIF cartridge with child Study barcode, visit, type of specimen and unique specimen barcode (e.g. BL\_350E\_STL\_00527999).
2. Place Xpert sample reagent, labelled Xpert MTB/RIF cartridge, 3ml plastic Pasteur pipettes, and the remainder of the processed pelleted specimen in the Biosafety Cabinet.
3. Label a NEW empty 50ml Falcon tube with Child Study barcode and specimen type (code) (e.g. "350E")
4. Pipet 1 ml of the pelleted specimen into the corresponding labelled 50ml Falcon tube.
5. Add 2 ml Xpert MTB/RIF sample reagent to each 1ml specimen in the 50ml Falcon tube (for a dilution ratio of 2:1) and invert 20 times (Do not vortex!).
6. Incubate for 15 min at room temperature and invert tubes 10 times after 8 min incubation.
7. Open the lid of the labelled Xpert MTB/RIF cartridge.
8. Transfer 2-3ml mixture completely into the corresponding labelled Xpert MTB/RIF cartridge, close the cartridge lid.
9. Carefully transport the Xpert MTB/RIF cartridge(s) to the GeneXpert laboratory, FISAN building, 5<sup>th</sup> floor, room F550 in a sealed container. Ensure that the cartridges are kept upright at ALL times. The Xpert cartridge must be run within 8 hours after opening.

#### 217. **GENEXPERT MEASUREMENT**

1. The following steps are performed in FISAN building room F5550
2. At all times wear disposable gloves.
3. Switch on GeneXpert machine and PC, start the GeneXpert Dx software and log in.
4. Click on "Create test" to start the measurement.
5. Click "Cancel" when prompted to scan a "Sample Barcode"
6. Scan the "cartridge barcode" when prompted
7. In the text box: Type in the specimen information from the Xpert cartridge (Study, visit, patient ID, specimen type; e.g. "PTB\_BL\_STL\_350E\_528999") and select "start test"
8. Load the cartridge into the indicated module (flashing light) and close the module gate.

9. Complete the dedicated sample log by filling in the amount of cartridges loaded into each Xpert machine.(Logs are on top of each Xpert machine)
10. Log out as the user WITHOUT closing or exiting the Xpert Software.
11. Each measurement requires 1 hour 55 minutes and is conducted automatically.
12. After the measurement is finalized the result can be viewed and archived by selecting the file in the “View results”.
13. Click the “Report” button, select “Generate Report File”, amend the file name, and save it to USB flash drive.
14. Optional: Open the pdf-file by clicking on “Preview pdf” and print the result page.
15. Close the software, shut down the PC, and switch off the instrument.
16. Discard spent cartridges in the yellow biohazardous waste container.

## 218. PROCESSING OF POSITIVE CULTURES

Every MGIT POSITIVE tube has a ZN smear and TB Ag MPT64 antigen test done. *Table 19* explains culture positive result interpretation and further testing

**Table 10.19. Interpretation of culture positive specimen results**

MGIT	ZN	TB Ag MPT64	Further Processing	HAIN MTBDR Test	HAIN CM	Final Result Reporting
+	+(Cording)	+/-	NONE	YES	Only if MTBDR negative	POSITIVE and TTP OR NTM
+	+(Not Cords)	+/-	NTM testing	NO*	YES	NTM OR POSITIVE and TTP
+	-	+	NONE	YES	NO	POSITIVE and TTP OR CONTAMINATED and TTP
+	-	-	NONE	NO	NO	CONTAMINATED and TTP
-	N/A	NONE	IF VISIBLE GROWTH	NONE	NONE	NONE

**219. ZIEHL NEELSEN (ZN) SMEAR MICROSCOPY**

1. Label glass slides with the corresponding Subject barcode (PID) and unique lab specimen barcode (-80°C barcode) and sample type.
2. Working inside the biosafety cabinet
3. Place 1 drop of TB precipitating fluid/fixative (NHLS fridge) on a slide.
4. Add 1 drop ( $\pm$  0.1ml) from the bottom of the positive MGIT culture tube and mix.
5. The required smear size is between 1.5 x 1.5 cm and 2.0 x 2.0 cm.
6. Carefully place slide on a hot tray (80°C) and allow to dry for at least 2 hours (heat kill) before staining.
7. Working inside the fume hood (NHLS)
8. Flood the slides with carbol fuchsin stain
9. Gently heat the slides from the bottom until steam rises for 1 minute. Do not boil
10. After 4 minutes rinse the carbol fuchsin off with tap water
11. Flood the slides with 3% acid alcohol solution for 2 minutes
12. Rinse with tap water
13. Flood slides with methylene blue counterstain for 2 minutes
14. Rinse with tap water
15. Air dry. Do not blot
16. Examine at least 100 fields per slide under a light microscope using the 100X lens with immersion oil

**220. RAPID TB AG MPT64 ANTIGEN TEST (BIOLINE)**

6. Processed according to package insert
7. Remove required amount of devices from the foil pouch and number it.
8. Working inside the biosafety cabinet, Add 100 $\mu$ l of liquid culture with a pipette into the sample well.
9. As the test begins to work, a purple colour moves across the result window in the centre of the test device.
10. Interpret the test result 15 minutes after sample application

**221. HAIN MTBDR TEST**

1. If positive cultures require the HAIN MTBDR test (see *Table 19*) then

2. Get the patient details for the specific specimen from the treatment response database (nursing team/blue patient file/hospital number)
3. Take the information to NHLS specimen reception and load the test type (PCR\_CU) for the specific specimen.
4. Label the corresponding MGIT tube with the patient barcode provided by reception
5. Take the labelled positive MGIT culture tube to the NHLS TB lab and ask the technologist on duty to add the specimen to the next batch of positive specimens to be tested for HAIN MTBDR

#### 222. **HAIN COMMON MYCOBACTERIA (CM)**

1. If positive cultures require the HAIN CM test (see *Table 19*) then
2. Get the patient details for the specific specimen from the treatment response database (nursing team/blue patient file/hospital number)
3. Take the information to NHLS specimen reception and load the test type (PCR\_CM) for the specific specimen.
4. Label the corresponding MGIT tube with the patient barcode provided by reception
5. Take the labelled positive MGIT culture tube to the NHLS TB lab and ask the technologist on duty to add the specimen to the next batch of positive specimens to be tested for HAIN CM.

#### 223. **RESULT REPORTING AND INTERPRETATION**

All lab results (smear, culture, Xpert and DST) are recorded in the lab log book and in the lab results excel spreadsheet (labPC; filename: "RXRESPONSE\_RESULTS\_ALL\_YYYY\_MM\_DD")

#### 224. **BACTEC MGIT 960 SYSTEM (CULTURE)**

1. The BACTEC system will record MGIT tubes with NO growth (No change in tube fluorescence) after 42 days as NEGATIVE.
2. The BACTEC system will record MGIT tubes with sufficient organism growth (significant change in tube fluorescence) as POSITIVE and will provide a time to positivity (TTP).
3. NEGATIVE and POSITIVE culture reports will be printed separately on the BACTEC system.
4. Record results in lab log book and in the results excel sheet according to Table 20

**Table 10.20. MGIT result reporting**

MGIT RESULT			
MGIT	Tube Growth	ZN	TB Ag MPt64
NEGATIVE	No visible growth	not done	not done
NEGATIVE	Visible growth	done	done
POSITIVE	Visible growth	done	done

**225. AURAMINE O STAIN AND MICROSCOPY**

1. View stained slides under a fluorescent microscope and record results according to WHO/IUTLD standardized guidelines (Table 21) in the lab log book and in the lab results excel spreadsheet.
2. Slides are read with the 40x objective microscope lens
3. Acid-fast bacilli emit a bright yellow fluorescence whilst cellular debris will be pale yellow in colour.
4. Record results in lab log book and in the results excel sheet

**Table 10.21. Reporting of Auramine smear microscopy results**

AURAMINE (AUR)		
No of AFB	Fields (40x objective)	Result Reporting
None	100	Negative for AFB
4-39	whole Slide	Scanty plus number of AFB
40-399	100	1+
4-40	1	2+
40+	1	3+

**226. GENEXPERT MEASUREMENT**

1. The reporting of Xpert results is done by email from the CCTR Laboratory as per their procedure.
2. Results are recorded according to Table 22
3. Record results in lab log book and in the results excel sheet

**Table 10.22. GeneXpert result options**

<b>Xpert Results</b>		
<b>Result</b>	<b>Frequency</b>	<b>RIF</b>
ERROR		
INVALID		
NEGATIVE		
POSITIVE	Very Low	Indeterminate
	Low	Sensitive
	Medium	Resistant
	High	
	Very High	

**227. ZIEHL-NEELSEN (ZN) SMEAR MICROSCOPY**

1. Report ZN results according to Table 23

**Table 10.23. ZN result reporting**

<b>Ziehl-Neelsen (ZN)</b>				
<b>No of AFB</b>	<b>Fields (100x objective)</b>	<b>Cording</b>	<b>Result Reporting</b>	<b>Further Testing</b>
None	100	N/A	Negative for AFB	TB Ag MPT64



1+ AFB	100	Cords	Positive for AFB; Cording	HAIN MTBDR
1+ AFB	101	Not Cords	Positive for AFB; Possible NTM	HAIN CM and HAIN AS

#### 228. **RAPID TB AG MPT64 ANTIGEN TEST (BIOLINE)**

1. Control band – coloured band on the left section of the result window, which indicates that the test is working properly.
2. Test band – coloured band on the right section of the result window, which indicates that the test is positive.
3. Negative result – The presence of the control band (C) only.
4. Positive result – The presence of both the control band (C) and the test band (T).
5. Invalid result – The absence of the control and (C) indicates that the test is invalid.

#### 229. **HAIN MTBDR TEST**

1. HAIN MTBDR test results will be emailed to the clinical team from NHLS
2. Report results in the lab log book and on the excel spreadsheet according to Table 24

**Table 10.24. HAIN MTBDR reporting**

<b>HAIN MTBDR Results</b>						
<b>MTBC</b>	<b>RIF</b>	<b>INH</b>	<b>2nd line tests</b>	<b>NTM ID</b>	<b>LIS Reporting 1st line</b>	<b>LIS Reporting 2nd line</b>
Negative	N/A	N/A	N/A	YES	NTM - species	N/A
Positive	-	-	NONE	NO	MTB POS; RIF sensitive; INH sensitive	N/A

Positive	+	-	YES	NO	MTB POS; RIF resistant; INH sensitive	Ofloxacin and Amikacin
Positive	-	+	YES	NO	MTB POS; RIF sensitive; INH resistant	Ofloxacin and Amikacin
Positive	+	+	YES	NO	MTB POS; RIF resistant; INH resistant	Ofloxacin and Amikacin

### 230. TROUBLESHOOTING

In case the GeneXpert instrument reports an error or an invalid result: Fetch the stored pellet specimen and repeat sample preparation and measurement as above.

In case the cartridge is dropped or falls over and a spillage occurs: Clean the spillage, discard the cartridge, fetch the stored pellet specimen, and repeat sample preparation and measurement as above.

### 231. REVIEW AND REVISION

This SOP was in use from March 2013 until June 2014.

### 232. REFERENCES

1. Siddiqi SH, Rüsç-Gerdes S. For BACTEC™ MGIT 960™ TB System (Also applicable for Manual MGIT). 2006;
2. Lawn SD, Nicol MP. Xpert® MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. *Future Microbiol.* 2011;6(9):1067–82.

## **B.16 Standard Operating Procedure 16: Detection of *Mycobacterium tuberculosis* in stool samples in children by GeneXpert MTB/RIF**

### **233. BACKGROUND**

234. The genexpert (xpert) mtb/rif instrument detects the *rpob* gene from *m. Tuberculosis* complex by using real time pcr and 5 fluorescent dna probes. Specimens for measurements are mixed and inactivated with provided sample reagent and pipetted into xpert mtb/rif cartridges. These cartridges are loaded into the instrument, processed automatically and analysed by the software (1).

### **235. OBJECTIVES**

Describes the process to detect *Mycobacterium tuberculosis* in stool specimens from children suspected to have pulmonary tuberculosis by using the GeneXpert MTB/RIF real time PCR platform.

### **236. MATERIALS AND EQUIPMENT**

At NHLS TB Laboratory:

1. Disposable and sterile 50 ml plastic tubes (Falcon Tube).
2. Phosphate buffer (PBS, pH 6.8, 0.037 M)
3. Refrigerated centrifuge with a minimum of 3000 x g (Centrifugal force)
4. Vortex mixer
5. Waste bag
6. Plastic Pasteur pipettes
7. Timer
8. Laboratory Coat
9. Disposable gloves
10. 70% Alcohol (or Ethanol or EtOH)
11. Biosafety Cabinet Class II
12. Specimen rack
13. Marker
14. N95 Mask

15. Scale (Adam AFP-1200L, max 1200g, smallest division = 10mg)
16. Xpert MTB/RIF cartridges
17. Xpert sample reagent

At TASK Laboratory Fisan building:

1. GeneXpert 4 module instrument connected to a PC with GeneXpert Dx software version 4.4a

### 237. SAFETY PRECAUTIONS

Standard safety operating procedures are to be followed at all times and all material should be treated as potentially infectious.

Before and after the specimens have been processed the laboratory work surface should be disinfected with 70% Alcohol.

All stool specimens are processed within the Biosafety Cabinet by lab technician (wearing of a fitted N95 mask is recommended).

### 238. HANDLING OF SPECIMEN IN THE CLINIC

1. Collect a fresh stool specimen from a paediatric source patient (study participant) into a sterile container as per stool collection SOP. The minimal mass is 0,5 g and should not exceed 5 g. The specimen will be collected in a 25 ml Faecal Cup with spoon container.
2. Each study participant has a Day 1 and Day 2 stool specimen that is split into two stool portions (maximum of 4 stool containers per participant; see *Table 25*)
3. Label the container and complete the appropriate forms
4. Place the container containing the stool in a transport bag with sleeve
5. The specimen must be kept in the fridge and transported at 2-8°C to the lab

**Table 10.25. Participant stool specimen breakdown**

Participant Stool Specimens		Stool Standard Xpert Protocol	Storage
Day 1	Portion 1	YES	overnight in fridge; 4°C
	Portion 2	NO	-20°C
Day 2	Portion 1	YES	overnight in fridge; 4°C

	Portion 2	NO	-20°C
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**239. SPECIMEN RECEPTION BY NURSING TEAM**

1. The nurse brings the specimens collected in the ward to room 0073 in their transport boxes until ready to scan.
2. See Lab Reception SOP for details on how to log the specimen in the database.
3. Specimens are placed in refrigerator 2-8°C

**240. DELAYS BETWEEN SPECIMEN COLLECTION AND SPECIMEN PROCESSING (PRINCIPAL)**

The Xpert manual specifies the following times between collection and processing for sputum samples (2) (stool samples not recommended for Xpert processing):

1. Sputum sediment: Store resuspended sediments at 2–8 °C for up to seven days.
2. Raw sputum: Transport and store specimens at 2–8 °C before processing whenever possible. If necessary, sputum specimens can be stored at a maximum of 35 °C for up to three days and then at 2–8 °C for an additional seven days.

**241. SPECIMEN RECEPTION BY LABORATORY TEAM (FOR LAB PERSONNEL ONLY)**

Specimens are collected by the Lab Tech and transported in a cold box to the NHLS lab on the 9<sup>th</sup> floor of Tygerberg Hospital (room 322) for processing.

Working inside the Biosafety Cabinet, remove specimens from bag and check that the specimens and barcodes match according to the REQ1 – DTTC Requisition Form that accompanies each Day1 and Day2 specimen.

**242. SPECIMEN PREPARATION**

1. The below steps are performed under a Biosafety Cabinet at NHLS in room 322 wearing a N95 mask.
2. Only open one lid at a time. Patient specimens must be processed independently to avoid a mix up. A maximum of 6 specimens can be processed at one time.
3. Process one specimen of each day (Day1: Portion 1 AND Day2: Portion 1) and indicate on REQ1 form which specimen (sample barcode) is processed.
4. Place remaining specimens of each day (Day1: Portion 2 AND Day2: Portion 2) back in sample transport bag and place in NHLS Lab fridge for temporary storage; move to -20°C freezer weekly (long term storage > 7 days at -20°C).

**243. CONCENTRATION**

1. Label a new 50 ml Falcon tube with child study barcode number and Day of specimen (e.g. "350-Day2").
2. Place an empty open 50 ml Falcon tube in a 4 tube specimen rack on the scale and press "zero"
3. Add a large scoop of specimen to the empty Falcon tube and weigh approximately 2 g (min.0.5 g– max 5 g).
4. Write the weight and Stool Appearance (see *Table 26*) on the Falcon tube (e.g. "2.45g – Solid") – Also record this information on the REQ1 form later
5. Add PBS buffer to the specimen(s) up to the 10 ml mark (Do one specimen at a time and avoid cross contamination)
6. Completely homogenise the specimen (in the PBS buffer) by Vortex mixing for approximately 20 seconds (or longer to ensure that specimen is thoroughly homogenised).
7. Place the homogenised specimen(s) and necessary counter balance (50 ml Falcon tube) into the centrifuge, close the lid.
8. Ensure that centrifuge is set at 3000 x g, 4°C and 20 min. Press Start.
9. After centrifugation, remove the 50 ml Falcon tubes containing the specimens and, inside the Biosafety Cabinet, decant the supernatant completely in the liquid-waste bucket.
10. Add PBS buffer to the concentrated specimen pellet(s) up to the 10 ml mark and vortex mix until completely homogenised.
11. Pulse (short centrifuge spin) the 50 ml Falcon tubes containing the homogenised specimens in the centrifuge to settle large debris particles: Start the centrifuge (same settings as before) when the centrifuge reaches 2000 x g (about 30 seconds), press the stop button.
12. After centrifugation, remove the 50 ml Falcon tubes containing the specimens and carefully place them inside the Biosafety Cabinet in a specimen rack (handle specimens carefully to avoid resuspension of the pelleted debris)

**Table 10.26. Stool Appearance Scale**

<b>Stool Appearance</b>	<b>Description</b>
Solid	Hard and difficult to break with a scoop

semi solid	Breaks off easily with a scoop
sticky	Sticks to the sides and scoop
liquid	Watery; Can be poured over

244.

245. **SAMPLE PREPARATION FOR GENEXPERT**

1. Label an Xpert MTB/RIF cartridge with Child Study barcode, day of specimen - day1 (E1) or day2 (E2) and unique specimen barcode (e.g. 350E2\_STL2\_00527999).
2. Place Xpert sample reagent, labelled Xpert MTB/RIF cartridge, 3 ml plastic Pasteur pipettes, and the processed pelleted specimen in the Biosafety Cabinet.
3. Label a NEW empty 50 ml Falcon tube with Child Study barcode and day of specimen (e.g. "350E2")
4. Pipet 1 ml from the middle layer (the layer between the settled and floating debris) from the briefly-centrifuged pelleted specimen into the corresponding labelled 50 ml Falcon tube from step 3 (avoid large particles as it clogs the cartridge filter).
5. Add 2 ml Xpert MTB/RIF sample reagent to each 1 ml specimen in the 50 ml Falcon tube (for a ratio of 2:1) and invert 20 times (Don't vortex!).
6. Incubate for 15 min at room temperature (invert tub 10 times after 8 min incubation).
7. Open the lid of the labelled Xpert MTB/RIF cartridge.
8. Transfer the 3 ml mixture completely into the corresponding labelled Xpert MTB/RIF cartridge, close the cartridge lid.
9. Carefully transport the Xpert MTB/RIF cartridge(s) to the GeneXpert laboratory, FISAN building, 5<sup>th</sup> floor, room F550 in a sealed container. Ensure that the cartridges are kept upright at ALL times. The Xpert cartridge must be used within 8 hours after opening.

246. **GENEXPERT MEASUREMENT**

1. The following steps are performed in FISAN building room F5550
2. At all times wear disposable gloves.
3. Switch on GeneXpert machine and PC, start the GeneXpert Dx software and log in.
4. Click on "Create test" to start the measurement.
5. Click "Cancel" when prompted to scan a "Sample Barcode"
6. Scan the "cartridge barcode" when prompted
7. In the text box: Type in the specimen information from the Xpert cartridge (time point, sample type, patient ID; e.g. "PTB\_BL\_STL2\_350E2\_528999") and "start test"

8. Load the cartridge into the indicated module (flashing light) and close the module gate.
9. Complete the dedicated sample log by filling in the amount of cartridges loaded into each Xpert machine (Logs are on top of each Xpert machine).
10. Log-out as the user WITHOUT closing or exiting the Xpert Software.
11. Each measurement requires 1 hour 55 minutes and is conducted automatically.
12. After the measurement is finalized the result can be viewed and archived by selecting the file in the “View results”.
13. Click the “Report” button, select “Generate Report File”, amend the file name, and save it to USB flash drive.
14. Optional: Open the pdf-file by clicking on “Preview pdf” and print the result page.
15. Close the software, shut down the PC, and switch off the instrument.
16. Discard spent cartridges in the yellow biohazardous waste container.

#### 247. **REPORTING**

The reporting of Xpert results is done by the CCTR Laboratory as per their procedure.

#### 248. **TROUBLESHOOTING**

In case the GeneXpert instrument reports an error or an invalid result: Fetch the stored pellet specimen and repeat sample preparation and measurement as above.

In case the cartridge is dropped or falls over and a spillage occurs: Clean the spillage, discard the cartridge, fetch the stored pellet specimen, and repeat sample preparation and measurement as above.

#### 249. **REVIEW AND REVISION**

This SOP was in use from June 2014 until study end

#### 250. **REFERENCES**

1. Lawn SD, Nicol MP. Xpert® MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. *Future Microbiol.* 2011;6(9):1067–82.
2. Cepheid®. Xpert® MTB/RIF Package Insert Ref GXMTB/RIF-US-10. 2015;(Feb):1–48. Available from: <http://www.cepheid.com/manageddownloads/xpert-mtb-rif-english-package-insert-301-1404-rev-b-february-2015.pdf>.



## Appendix C

### Consent documents

#### C.1 Information leaflet for study: “Diagnostic yield and response to treatment in childhood intra-thoracic Tuberculosis”

Health Research Ethics Committee Protocol Number N11/09/282

Dear Parent/Guardian

Your child is being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this research. Please ask the study staff any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research means and how your child will be involved. Also, your child’s participation is **entirely voluntary** which means that you are free to decline (say “no”) to having your child participate. If you say no, this will not affect you or your child negatively in any way whatsoever. You are also free to withdraw your child from the study at any point, even if initially agreed to your child take part.

This study has been approved by the **Human Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research. We are planning to have 450 children in this study, 225 with TB and 225 without TB. We estimate that 80-100 children will be HIV-infected.

#### ***Who is doing this research?***

The main researcher is **Dr Elisabetta Walters**, a paediatrician working at the Desmond Tutu TB Centre at Stellenbosch University. At this research centre we learn more about TB in children. The contact number for Dr E. Walters is **021-9389110**. You may also contact the **Human Research Ethics Committee** at Stellenbosch University if you are not satisfied with the information given to you: the contact person is Elvira Rohland at 021 938 9677.

#### **Why has your child been invited to participate?**

We have invited you and your child to take part in the research study because your child came to hospital and may have TB and needs to be tested for TB.

### ***What is TB?***

Tuberculosis (TB) is an infectious disease caused by germs (bacteria) spread by coughing. TB mainly affects the lungs, but can also affect other parts of the body like the brain (TB meningitis) and lymph glands. Although both adults and children can suffer from TB, children often get more serious disease. Children younger than five years of age are at very high risk of getting TB and serious forms of the disease. Children who have weakened immune systems, such as children with HIV infection and with poor nutrition, are at even higher risk of getting TB. We are therefore looking at children 13 years of age and younger in this research. We will include children with HIV infection in this study since the diagnosis of TB in HIV-positive children is especially difficult to make.

### ***What is our research study about?***

Our research study tries to answer 2 questions:

1. How well do 3 new tests to diagnose (find) TB in children work?
2. How do children respond to TB treatment?

#### ***1. The new TB tests we are evaluating:***

The diagnosis of TB in children is usually made with tuberculin skin tests (injection under the skin), chest films (x-rays), and asking about symptoms such as ongoing cough or weight loss, a history of contact with an adult with TB. In adults, it is much easier to diagnose TB, because adults can cough up sputum (phlegm), which can be sent to the laboratory for diagnosis. Small children swallow their sputum, and that is why, when we think that a child has TB in her/his lungs, we try to get sputum from the child by putting a nasogastric tube (a stomach tube) into the stomach to aspirate (pull out) some swallowed sputum. This procedure is called **gastric aspiration** or gastric washing. We can also nebulise the child with a special mask connected to some oxygen and salt solution. This can make the child cough, so that we can suck out some of the coughed up sputum from the back of the throat with a soft tube. This procedure is called **induced sputum**. Sometimes TB can be picked up from the mucus in the child's nose. By putting 2 drops of water into the child's nostrils, the mucus can be sucked out and tested for TB. We call this **nasopharyngeal aspiration**. At the laboratory these samples are examined under a microscope (this is called **TB smear**) and then processed until a final result is available (this is called **TB culture**). Results usually take about 6 weeks. Although the laboratory tests on gastric aspirate and induced sputum are the best we

currently have available and are necessary to prove that a child has TB, the available TB tests have some problems in young children:

1. It is not easy to get these sputum samples from children. Children have to be on an empty stomach for these tests and, although they are not dangerous or painful, they are a little bit uncomfortable for the child.
2. These tests can be negative even though the child has TB. In general, out of 10 children with TB, only 3-4 children will have a positive TB culture and only 1 will have a positive smear.
3. The results take a long time and are expensive.

Therefore, we want to evaluate (test out) new TB tests in children. We also want to try new ways of getting sputum from children.

These new tests are called the **GeneXpert**, **Genedrive** and the **urine LAM** tests.

The **GeneXpert and Genedrive tests** can be done on sputum, gastric aspirate and stool. The test is done by putting a sample into a specialized machine, which can pick up very small numbers of TB germs without having to do the TB culture. The results are available in 2 hours. The reason we want to try out the tests is that they are very fast. We also want to see how well they can pick up TB from gastric aspirates and stool samples.

**Urine LAM** is a dipstick test (like a pregnancy test) which picks up part of the TB germ (a TB protein) in the urine. The reason we want to try out this test is that it is very fast (a few minutes), urine is easy to get from children and the test is cheap.

We do not know very much at this stage about how these new tests work to diagnose TB in children. New samples we want to test to find out if the child has TB

1. The string test. We have mentioned above that the usual way of getting sputum from young children is to pull the sputum out of the stomach with a soft plastic tube after an overnight fast. Recently, research from South America has shown that another way of getting the sputum is to wrap a nylon string around the nasogastric tube and leaving it in the child's stomach overnight. As the child coughs and swallows the sputum, the string becomes soaked with sputum. When the tube is removed in the morning, the string can be sent to the lab to be tested for TB. In this way, 2 samples can be taken with one procedure: the string and the gastric aspirate. We want to try the string test in this research study.

We also plan to try out new ways of processing samples in the laboratory to improve our chance of finding TB. We want to put all the samples that we collect on one day together so that we have one big sample to test. We think that the big sample will have more TB germs. This will make it easier for us to pick up the TB germ if it there. Once we start trying out this new method, we will need 450 children to see if it works.

### ***Why we want to study how children respond to TB treatment***

Most of the information on how we should treat TB and check whether this treatment is working comes from research in **adults** with TB. Most adults with TB are treated for 6 months, and they are monitored (checked) by taking regular sputum samples for smear (examination under the microscope to look for TB germs). If the smear was positive in the beginning (meaning that TB germs were visible with the microscope) and becomes negative (no TB germs visible) during TB treatment, we know that the person is getting better. In children, smear is usually negative even before they start treatment. Sputum is therefore not so helpful in checking treatment response in children with TB. We also know that there are some other signs that can help us to decide if a TB patient may react on TB treatment. These include looking at the chest x-ray, and checking the amount of weight gained during treatment. We have very little information on how to measure these signs in children with TB. We need to look more closely at how children respond to TB treatment so that we can make good decisions about what treatment to give and for how long. This is important, because children with TB are different to adults with TB.

The things we want to look to help us decide how a child is responding to TB treatment are:

1. Symptoms, especially weight gain during treatment
2. Bacteriology (repeated smear and culture of the TB germ in gastric aspirate and induced sputum)
3. Chest X-rays
4. Blood tests that show how the body's immune system (the way the body protects itself) is responding to the TB germ.

### ***What will happen to your child if you give consent to participate in this study?***

Your child has been asked to take part in this study because the doctors in this hospital think that he/she may have TB. Your child will therefore have all the normal (routine) tests that are done when we think a child may have TB. These include 2 early morning gastric aspirates, a chest X-ray, a skin test and an HIV test. All of these tests are done routinely, regardless of whether your child would be part of a research study or not. One difference will be that together with the gastric aspirate, we will also collect sputum from the string that we will wrap around the nasogastric tube. We will ask you information about your child's symptoms (such as cough, loss of weight) and medical history.

A doctor will examine your child.

In addition to the routine tests, we will also take 2 induced sputum and 2 nasopharyngeal aspirate samples on 2 separate days. We will draw some blood (2 teaspoons), get urine with a urine bag or a container if the child can pass urine on his/her own, ask you to collect some stool into a container and maybe get some saliva (spit) by letting the child suck on some cotton wool.

Once we have collected all these samples, we will give you a date for a follow-up visit to come back to hospital.

### **When do follow-up visits happen?**

If your child is diagnosed with TB and starts TB treatment, we will follow your child regularly until the end of TB treatment. We will see your child at 2 weeks (if older than 5 years), one month and at 2 months from the start of TB treatment, and also at the end of treatment. We may also decide to see your child 6 months after this date, after TB treatment has been finished.

If your child is not started on TB treatment, we will give you a follow-up appointment 1, 2, 4 and 6 months after we have seen your child in hospital. At the 2-month visit, we will give you results of the routine TB tests and check how well your child is doing.

### ***If your child has TB, what will happen at the follow-up visits?***

At each visit, we will ask you about your child's symptoms and examine the child. We will also ask how well he/she is taking his/her treatment. We will ask you to bring the child's clinic card and TB treatment card from the clinic to every visit, and any medications the child is taking. If your child is HIV-positive, you should bring all your child's HIV treatments as well.

At the 1- month visit, we will take induced sputum and NPA to send to the TB laboratory. At the 2 and 6 month-visits, we will take a gastric aspirate, induced sputum, NPA, a chest x-ray and a small amount of blood and urine. We will take stool at every visit. Your child should be fasting (on an empty stomach) for 4 hours on the day of the follow-up visit, so we advise you not to give the child milk or breakfast in the morning. We will take the samples as soon as you arrive at the hospital and the child can get breakfast immediately afterwards. You will need to bring some food and drink for the child to have after the procedures are done.

### ***Where will the follow-up visits take place?***

We will see your child at the hospital where the child was first admitted. We will have a special room where we will see your child, and will let you know where to come before your child leaves the hospital.

### ***What are the potential risks and benefits associated with this study?***

#### **The risks or discomforts of drawing blood from your child:**

- Taking blood may cause your child some discomfort
- Some bleeding or bruising may occur where the needle enters the body

- Very rarely, infection at the puncture site may occur

We will limit these risks by using an experienced, skilled nurse or doctor to draw blood from your child and by following standard guidelines. Children will be comforted and distracted and 3 attempts at most will be made to obtain the blood sample. The total amount of blood that we will draw is safe and is less than the recommended maximum amount for research studies.

### **The risks or discomforts of taking gastric aspirates and induced sputum**

Gastric aspirates and induced sputum are done routinely to test for TB in children and they are very safe procedures. Sometimes, the child may have a nose bleed after the procedures. This usually stops as soon as the procedure is finished. We will make sure that it is safe for your child (according to his/her condition) to have these procedures before they are done. When a child has a nebulisation for sputum induction, the child will cough: this is what we expect, and it will allow the phlegm (sputum) to come up so that we can test it for TB. The coughing usually settles after a few minutes.

### **The risks or discomforts of Mantoux skin testing**

Complications of doing the TB skin test are usually mild and include pain or irritation at the site of injection immediately after injection. Severe complications of the TB skin tests are very rare, and include blistering and ulceration of the skin. Please contact the study nurse if your child complains of severe itching, pain or blistering of the skin. There is a cream available that can be applied to the skin to prevent complications of the skin test.

### **What will happen if your child develops a medical illness as a result of taking part in this study?**

We do not expect that your child will suffer any serious complications as a result of taking part in this study. If your child develops any serious medical problems please immediately report this to the study staff. The contact details of the study staff are on the first page of this leaflet. All treatment related to medical problems in your child will be provided free of charge.

### ***Confidentiality***

We will make every effort to keep your child's information private and confidential. This includes personal information and information about TB and HIV test results. The information we collect on your child will not be linked to your child's name or any other personal details in the computer database. Your child's information and stored samples will be coded (get a special number). Any paper records with your child's information will be stored in a locked cabinet and will not be accessible to anyone outside the study team. The documents with your child's name on will be stored separately from the documents that contain other information about your child. The risk of your information being disclosed as a result of this study is therefore likely to be minimal. There is also a risk that your child may be psychologically affected as a result of participating in this

study. A study counselor or nurse will be available for further counseling throughout the duration of the study if you are concerned about this.

### ***Potential benefits of participating in this study***

There is no direct or specific benefit of your child participating in this study. If your child has TB, he/she will be followed up very closely throughout the time of treatment. He/she will be seen by a doctor 3 times during this period of time. This will allow us to ensure that your child is taking the correct treatment, and we will also be able to look out for signs that he/she is not doing well. However, it remains the clinic/hospital doctor's responsibility to decide how to manage and treat your child.

The fact that your child is part of this research study will not affect the decision to treat your child for TB. The findings from this research will allow us to make some decisions about how useful these new tests are in general for children with TB. By allowing your child to take part in this project, it is possible that children in the larger community in the future may benefit from better tools to diagnose TB. The results of these new tests will not affect the way your child, specifically, is treated.

### ***Cost***

You will not receive any money for taking part in this study. We will however provide you with the transport money you need for study visits. If you are not able to arrange transport for study visits, we can help you with transport.

### ***Future use***

We will store some of your child's blood, urine, stool and saliva samples and any TB cultures for further TB testing including immunology (measuring the body's ability to fight the TB germ). We intend to store these samples for at least 5 years. If you give permission, we will do a genetic test on your child's blood to see if we can pick up the body's reaction to the TB germ. This could help to develop a new test for TB in the future that uses blood rather than sputum. We have more information about this test in a separate form. Your child's samples will be coded and stored at the Department of Medical Microbiology and the Department of Immunology, Tygerberg Campus, Stellenbosch University. Your child's specimens may be shared with other investigators, or with other institutions in a joint research project. We do not foresee any risks to the storage and future use of these stored specimens. We will not collect any extra blood for these stored samples. The results of extra tests done on these samples will not be stored in your child's medical records and will not be made available to your child's routine healthcare provider. None of the information that may be published related to your child's stored specimens will have your child's name or other personal identifiers. If you refuse permission for storage of your child's samples, we will store the samples but they will not be linked to your child in any way (they will be "anonymized").

### ***What happens if new information about these new tests or about TB in general is discovered***

***during the course of this research project?***

If new information is found, we will inform you and we will make sure that your child has the best care that is available. We will follow the National South African best practice guidelines and only continue or add tests that may improve care of children with TB.

***Summary of your child's rights as a participant in this research study***

Your permission for your child's participation in this research study is voluntary. The alternatives to participating in this study are to attend the local TB clinic only and not to come back to the research team. Refusing to participate will not change your child's usual health care or involve any penalty or loss of benefits to which your child is entitled. You may withdraw your child from participation at any time without it affecting your child's medical care. You can do this by telling someone on the study team that you would like to withdraw your child. You may also withdraw your child's specimens.

If your child is older than 7 years, we will ask the child to agree to participate and we will respect your child's opinion.

If information generated from this study is published or presented, your child's identity will not be disclosed. If new information becomes available that may affect the risks or benefits associated with this study or your willingness to let your child participate in it, you will be notified so that you can decide whether or not to let your child continue participating.



## C.2 Informed consent form

### STUDY TITLE: "DIAGNOSTIC YIELD AND RESPONSE TO TREATMENT IN CHILDHOOD INTRA- THORACIC TUBERCULOSIS"

- I understand what this research study is about.
- I understand that my child will have routine and additional (extra) investigations for TB.
- I give consent for my child to have an HIV test.
- I consent to the following additional investigations:
1. 2 induced sputa, nasopharyngeal aspirates and string tests, blood, urine, and a stool sample at the initial visit
  2. Induced sputum, nasopharyngeal aspirate and gastric aspirate tests at 1, 2 and 6 months if the child is started on TB treatment.
  3. A sample of blood at 2 and 6 months if the child is started on TB treatment.
  4. Stool and urine samples at every visit
  5. A chest x-ray at 2 and 6 months.
- I consent for my child to be given 2 puffs of salbutamol before the induced sputum procedure (except if there are contra-indications).
- I understand that samples from my child may be stored for future TB testing for at least 5 years. *If consent not given, samples will be anonymized.*
- I understand that all the information that I give about my child, and all the results of the tests will be kept confidential. My child's identifying details will not be linked to information or results obtained from my child.
- I understand that I will not receive any monetary compensation (money) for my child participating in this study.
- I understand that I can withdraw my child from the study at any time. If I do so, the care of my child will not be negatively affected.

**Surname (child):** \_\_\_\_\_ **Name:** \_\_\_\_\_ (please print)

**Date of Birth (dd/mm/yyyy):** \_\_\_/\_\_\_/\_\_\_\_ **Sex (M/F):** \_\_\_\_\_

**Surname (parent/guardian):** \_\_\_\_\_ **Name:** \_\_\_\_\_

Signed at (place) ..... on (date) \_\_\_/\_\_\_/\_\_\_ at  
(time) \_\_\_h\_\_\_min.

.....  
**Signature (or thumbprint) of parent/legal guardian**

.....  
**Signature of witness (only if parent gives thumbprint)**

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**Declaration by study personnel**

I (name) ....., designation (clinician, nurse, counsellor)  
..... declare that:

- I explained the information in this document to .....
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understand all aspects of the research, as discussed above.
- I did/did not use an interpreter (if an interpreter is used, then the interpreter must sign the declaration below).

Signed at (place) ..... on (date)\_\_\_\_\_/\_\_\_/\_\_\_ at (time)  
\_\_\_h\_\_\_min.

.....  
**Signature of study personnel**

**Declaration by interpreter**

I (*name*) ..... declare that:

- I assisted the investigator (*name*) ..... to explain the information in this document to (*name of parent/legal guardian*) ..... using the language medium of Afrikaans/isiXhosa.
- We encouraged him/her to ask questions and s/he took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the parent/legal guardian fully understands the content of this informed consent document and has had all his/her questions satisfactorily answered.

Signed at (*place*) ..... on (*date*) \_\_\_\_\_/\_\_\_/\_\_\_\_ at (time) \_\_\_\_\_h\_\_\_\_min.

.....

**Signature of interpreter**

	<p>STELLENBOSCH UNIVERSITY FACULTY OF HEALTH SCIENCES</p>	
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### C.3 Participant information leaflet and assent form



**RESEARCH PROJECT: NEW WAYS OF FINDING OUT IF CHILDREN HAVE TB**

**RESEARCHERS NAME: DR LIZ WALTERS**

**ADDRESS: DESMOND TUTU TB CENTRE, TYGERBERG HOSPITAL**

**CONTACT NUMBER: 021-9389110 or 021-9389746**

#### **What is RESEARCH?**

Research is something we do to find out how things (and people) work. We use research projects or studies to help us find out more about disease or illness. Research also helps us to find better ways of helping, or treating children who are sick.

#### **Why have I been invited to take part in this research project?**

You are in hospital because you are not well, and your doctor thinks it is good to check you out for TB.

#### **Who is doing the research?**

A group of doctors from the Desmond Tutu TB Centre at Tygerberg Hospital are doing the project. The head of the project is Dr Liz Walters. The nurses are Melvina Stollie and Ingrid Rowan, and Pamela Mvinjelwa and Asanda Mfazwe are our helpers.

#### **What is this research project all about?**

Many children in our city have TB. TB is an illness that makes children very sick. We have good medicines for treating TB and making children better again, but it is sometimes very difficult to know if a child has TB. TB germs can hide very well and can be difficult to find. We have some tests that help us to try and find out if children have TB, but many times we are still not sure. Our project is testing new ways of looking for TB in children.

When a person becomes sick with TB, the TB germ comes into the body and can spread to the chest (lungs), the blood, the tummy and the brain. It can also land up in the urine (wee wee) and poo.

We want to see where in your body we can find the TB germ. We also want to know if there are better ways of looking for the germ. It is good to find the TB germ if it is there, because then we can give you the right medicines.

Our body tries to fight the TB germ. Some bodies are stronger than others. Some people can fight the TB germ away, but some people can get very sick from TB because their body is weak. We are trying to understand why this happens.

We can do this by taking a small bit of blood from you.

We would also like to do a blood test for HIV if you have never been tested for HIV before. HIV is another infection that is very common in South Africa.

HIV is a germ that makes the body weak. People who have the HIV germ get sick often and can pick up TB much more easily than people who don't have HIV. Testing for HIV is easy and we have good treatment for people who have HIV so that they can feel strong again.

If our new TB tests work well, it will be easier to know if a child who is sick has TB. So we will be able to start the right treatment quickly and make the child better faster.

We would also like to keep some of the spit, blood, wee and poo in a special fridge, so that we can use it later on if we think of more tests that will help us find TB.

### **What will happen to me in this study?**

Most of the tests that will be done are the same tests that your doctor would do to see if you have TB. We will ask some questions about your illness and a doctor will examine you. You will have a chest Xray (a picture of your chest) done, and we will try and take some of your spit to test for TB. If you agree to be part of our project, we will ask if we can take a blood test and some wee and poo from you. Taking blood can feel a little bit painful, like a prick on the skin, but it doesn't last long and we will only take 2 teaspoons of blood. We will take the spit, blood, wee and poo to see if find the TB germ. We will test the blood for HIV if you say it's ok. We will

keep a little of all the samples in a fridge to store for later on if we come up with new tests for TB.

### **Can anything bad happen to me?**

Taking a blood test can cause a little bit of pain at first, but it should go away quickly. Sometimes a bit of bleeding or a small bruise can happen where the blood is drawn but that will also go away quickly.

### **Can anything good happen to me?**

If we find out you have TB we will let you and your doctor know so that you can get treatment as soon as possible and get better again.

By being part of our project, you will help us find better tests for TB than the ones we have now. That will help us to treat children with TB earlier so that they get well faster.

### **Will anyone know I am in the study?**

Only the project team will know that you are part of the study. We will not let anyone else know any of the information we have about you.

### **Who can I talk to about the study?**



If you have any questions about the project, or any problems with it, you should first speak to your mother/father or the person who cares for you and who brought you to the hospital, so that they can call one of us.

Our numbers are:

Dr Liz Walters at 021-XXXXX

Sr Melvina Stollie at 021-XXXX

Pam Mvinjelwa 082XXXXX

### **What if I do not want to do this?**

You don't have to agree to be part of this project if you don't want to. You will not get into any trouble for saying no. You will still be looked after by your doctor. If you decide to help us in our project but later on you want to stop, you will be free to stop at any time.

**Do you understand this research study?**

**Will you take part in it?**

**Have the doctor and nurse answered all your questions?**

**Do you understand that you can pull out of the study at any time?**

\_\_\_\_\_  
**Signature of Child**                      **Date**                      **Name of child**  
**(print)**

\_\_\_\_\_  
**Signature of researcher**                      **Date**                      **RA Code**

report forms

TREATMENT RESPONSE STUDY CRF-B POOLING – CASE REPORT FORM - BASELINE											
DATE	D	D	M	M	Y	Y	Y	Y		<div style="border: 1px solid black; width: 100%; height: 100%; margin-bottom: 5px;"></div> <p style="text-align: center; margin: 0;">CHILD BARCODE</p>	
TIME					H						
<p>** Compulsory question</p> <p>* Compulsory question depending on parent question</p> <p>Make use of jump instructions</p> <p>Make use of a black pen only</p>											
<b>A DEMOGRAPHIC DATA</b>											
A_1	Date of Birth	D	D	M	M	Y	Y	Y	Y	**	
A_2	Sex	1 - Male		2 - Female							**
A_3	Race	1	Black							**	
		2	Coloured								
		3	Indian								
		4	White								
A_4	Home language	1	English							**	
		2	Afrikaans								
		3	Xhosa								
		4	Sotho								
		5	Zulu								
		-8	Other								
		Specify									
A_5	Household size	Adults >17 years									**
		Children 6-17 Years									**
		Children 2-5 years									
		Infants <2 years									
A_6	Household income (put total number in box)	Social grants/pension									**
		Employed adults: casual									
		Employed adults: permanent									
A_7	Who is primary caregiver (tick one)	Mother									**
		Father									
		Grandparent									
		Other family									
		Other community member, not fostering									
		Place of safety (children's home)									
		Foster care									
		Adoptive parent									
PAGE 1 OF 16										TREATMENT RESPONSE STUDY	



**TREATMENT RESPONSE STUDY**  
**CRF-B POOLING – CASE REPORT FORM - BASELINE**

A_8	Highest education level of primary caregiver	1	No formal education		**
		2	Primary school		
		3	High school		
		4	Diploma		
		5	Degree		
		6	Informal qualifications/training		

A-9	Basic household services (>1 may apply)	1	Electricity	0 - No	1 - Yes	**
		1.1	Working fridge	0 - No	1 - Yes	
		1.2	Electric or gas stove	0 - No	1 - Yes	
		2	Paraffin stove	0 - No	1 - Yes	
		3	Water	0 - No	1 - Yes	
		3.1	Inside tap	0 - No	1 - Yes	
		3.2	Outside tap	0 - No	1 - Yes	
		4	Toilet	0 - No	1 - Yes	
		4.1	Inside toilet	0 - No	1 - Yes	
		4.2	Outside toilet	0 - No	1 - Yes	
		4.3	Bucket system	0 - No	1 - Yes	

A-10	Type of housing	1	Formal (brick)		**
		1.1	Number of rooms excl toilet		
		2	Informal (shack)		
		3	Wendy house/bungalow		
		4	Shelter		
		5	No housing (on the street)		

B_1	Cough	0 - No	1 - Yes	-5 - Unknown	**	If No, then Go to B_2
		days				
B_1_1	If yes, duration of cough					
B_1_2	Cough pattern	1.Acute (<14d)	2.Prolonged (≥14d)	3.Recurrent		If Acute, go to B_1_3

B_1_2_1	If prolonged or recurrent, is cough associated with:	B_1_2_1_1	Exertion	0 - No	1 - Yes	-5 - Unknown	*
		B_1_2_1_2	Feeding	0 - No	1 - Yes	-5 - Unknown	
		B_1_2_1_3	Excitement	0 - No	1 - Yes	-5 - Unknown	
		B_1_2_1_4	Allergens (e.g. smoke/dust)	0 - No	1 - Yes	-5 - Unknown	
		B_1_2_1_5	Night-time	0 - No	1 - Yes	-5 - Unknown	
		B_1_2_1_6	Upper respiratory tract infections	0 - No	1 - Yes	-5 - Unknown	
		B_1_2_1_7	Other	0 - No	1 - Yes	-5 - Unknown	
		B_1_2_1_7_1	Specify				

B_1_3	Day to day variation of cough	1	Everyday	*
		2	Every second day	
		3	Occasional	

B_1_4	Character	1	Mostly wet	*
		2	Mostly dry	

**TREATMENT RESPONSE STUDY**  
**CRF-B POOLING – CASE REPORT FORM - BASELINE**

<b>B_2</b>	<b>Wheezing</b>	0 - No	1 - Yes	-5 - Unknown	
<b>B_2_1</b>	If yes, duration of wheezing				days
<b>B_2_2</b>	<b>Pattern of wheezing</b>	1.Acute (<14d)	2.Prolonged (≥14d)	3.Recurrent	If Acute, go to B_3
<b>B_2_2_1</b>	If prolonged or recurrent, is wheeze associated with:				
<b>B_2_2_1_1</b>	Exertion	0 - No	1 - Yes	-5 - Unknown	
<b>B_2_2_1_2</b>	Feeding	0 - No	1 - Yes	-5 - Unknown	
<b>B_2_2_1_3</b>	Excitement	0 - No	1 - Yes	-5 - Unknown	
<b>B_2_2_1_4</b>	Allergens (e.g. smoke/dust)	0 - No	1 - Yes	-5 - Unknown	
<b>B_2_2_1_5</b>	Night-time	0 - No	1 - Yes	-5 - Unknown	
<b>B_2_2_1_6</b>	Upper respiratory tract infections	0 - No	1 - Yes	-5 - Unknown	
<b>B_2_2_1_7</b>	Other	0 - No	1 - Yes	-5 - Unknown	
	<b>B_2_2_1_7_1</b> If other, specify				
<b>B_3</b>	<b>Fever</b>	0 - No	1 - Yes	-5 - Unknown	** If No, then Go to B_4
	If yes, duration of fever:				
<b>B_3_1</b>	Before hospital admission				days
<b>B_3_2</b>	Since hospital admission				days
<b>B_3_3</b>	Variation of fever	1	Daily		*
		2	Intermittent		
<b>B_3_4</b>	Highest recorded temperature				°C *
<b>B_3_5</b>	Site of thermometer	1	Axillary		*
		2	Oral		
		3	Rectal		
		4	Ear		
<b>B_4</b>	<b>Lack of appetite for solids or liquids</b>	0 - No	1 - Yes	-5 - Unknown	** If No, then Go to B_5
<b>B_4_1</b>	Lack of appetite for solids?	0 - No	1 - Yes	N/A	*
<b>B_4_1_1</b>	If yes, has child always been a picky eater?	0 - No	1 - Yes		** If No, then Go to B_4_2
<b>B_4_1_2</b>	If not a picky eater, for how long has child had poor appetite?				days
<b>B_4_2</b>	Lack of appetite for liquids	0 - No	1 - Yes		*
<b>B_5</b>	Diminished playfulness	0 - No	1 - Yes	-5 - Unknown	**
<b>B_6</b>	Lethargy (sleepiness)	0 - No	1 - Yes	-5 - Unknown	** If Yes, specify below. If no, go to B_7
<b>B_6_1</b>	Awake in the day but not engaging in usual activities	0 - No	1 - Yes	-5 - Unknown	*
<b>B_6_2</b>	Mostly asleep / lying down	0 - No	1 - Yes	-5 - Unknown	*
<b>B_7</b>	Is child currently 2 months old or older?	0 - No	1 - Yes		** If Yes, then go to B_8
<b>B_7_1</b>	Neonatal pneumonia not responding to appropriate course of inpatient antibiotics and/or antivirals	0 - No	1 - Yes	-5 - Unknown	*
<b>B_7_2</b>	Unexplained unresponsive sepsis-like syndrome (see results section)	0 - No	1 - Yes	-5 - Unknown	*
<b>B_8</b>	Other symptoms?	0 - No	1 - Yes		** If No, go to C_1

**TREATMENT RESPONSE STUDY  
CRF-B POOLING – CASE REPORT FORM - BASELINE**

Specify	Symptom	Duration (d)
B_8_1		
B_8_2		
B_8_3		
B_8_4		

C_1	Any documented previous TB episode or treatment	0 - No	1 - Yes	-5 - Unknown	**	If No/Unkn Go to D1		
C_1_1	If yes, how many?	1 One	2 Two	3 Three	4 Four	-5 Unknown		
C_1_2	Bacteriological status of most recent episode	1 Smear positive	2 Smear negative	3 Smear unknown	4 Culture/Xpert positive	5 Culture/Xpert negative	6 Culture/Xpert unknown	More than 1 can apply
C_1_3	If yes, state outcome of most recent previous TB episode	1 Cured	2 Treatment Completed	3 Defaulted	4 Treatment Failure	5 Transferred Out	-5 Unknown	
C_1_4	Date of Outcome	M M Y Y Y Y						
C_1_5	Site of previous TB episode (>1 can apply)	1 Intrathoracic (incl mediastinal nodes, parenchymal disease, pleural TB)						
		2 Meningitis						
		3 Abdominal						
		4 Miliary						
		5 Lymphadenitis						
		-5 Unknown						
		-8 Other						
		Specify Other						

D_1	Does the child <b>currently</b> have a TB contact?	0 - No	1 - Yes	-5 - Unknown	**	If No/Unkn D_2
D_1_1	If yes, how many TB contacts? <i>*Complete a TB contact form for each</i>	1 One	2 Two	3 Three	4 Four or more	

**TREATMENT RESPONSE STUDY**  
**CRF-B POOLING – CASE REPORT FORM - BASELINE**

D\_2 Has the child had any contact with a Confirmed or Suspected TB index case in the last **0-12 months**? 

0 – No	1 – Yes	-5 – Unknown
--------	---------	--------------

 \*\* If No/Unkn Go to E\_1

D\_2\_1 If yes, how many TB contacts?  
*\*Complete a TB contact form for each*

1	One
2	Two
3	Three
4	Four or more

Answer the following questions for the most significant contact:

D\_3 Is the index case currently coughing? 

1-Yes	0-No	-5-Unk
-------	------	--------

 \*

D\_4 What is the relation of the TB index case to the child?  

1 Mother	2 Father
3 Grandmother	4 Grandfather
5 Aunt	6 Uncle
7 Cousin	8 Sibling
9 Neighbour	10 Lodger
11 Caregiver other than family	-8 Other

 \*

D\_5 Is the index case the child's primary care giver? 

1-Yes	0-No	-5-Unk
-------	------	--------

 \*

D\_6 Is there daily contact between the child and index case? 

1-Yes	0-No	-5-Unk
-------	------	--------

 \*

D\_6\_1 Where does the TB index case live?  

1	In the house
2	Neighbouring house/same plot (yard)
-8	Other

 \*

D\_6\_2 Does the index case sleep in the same room as child? 

0 - No	1 – Yes	-5 Unknown
--------	---------	------------

 \*

D\_6\_3 Does the index case sleep in the same bed as the child? 

0 - No	1 – Yes	-5 Unknown
--------	---------	------------

 \*

D\_6\_4 How many hours on average does the index case spend with the child per day?  

1	0 – 4 Hrs
2	5 – 8 Hrs
3	9 – 12 Hrs
4	> 12 Hrs

 \*

D\_7 Did the child start IPT for this contact episode? 

0 - No	1 – Yes	-5 Unknown
--------	---------	------------

 \*

D\_7\_1 If yes, date 

M	M	Y	Y	Y	Y
---	---	---	---	---	---

 \*

D\_7\_2 At which clinic 

--

D\_8 In infants <2 months, also any one of:  

D_8_1	Maternal TB diagnosed in third trimester	0 - No	1 – Yes	-5 Unkn	-4 N/A
D_8_2	Maternal TB diagnosed any time after delivery of child	0 - No	1 – Yes	-5 Unkn	-4 N/A
D_8_3	Confirmed maternal endometrial TB on histology and/or culture	0 - No	1 – Yes	-5 Unkn	-4 N/A

E1\_1 Has the child had any perinatal HIV exposure? 

0 - No	1 – Yes	-5 Unknown
--------	---------	------------

 \*\* If No/ Unk, then Go to E2\_1

**TREATMENT RESPONSE STUDY**  
**CRF-B POOLING – CASE REPORT FORM - BASELINE**

E1_1_1	Adequate antenatal PMTCT (from 28 weeks)	0 - No	1 - Yes	-5 Unknown	*																														
E1_1_2	Postnatal PMTCT (NVP for 6w)	0 - No	1 - Yes	-5 Unknown	*																														
E1_1_3	Feeding method for first 6 months	1 - Breast	2 - Formula	3 - Mixed	*																														
E1_1_4	HIV PCR	0 - No	1 - Yes	-5 Unknown	*																														
E1_1_4_1	<input type="text"/> Early (6w)	1. Date	D D M M Y Y Y Y		*																														
		2.Result	0 - Negative 1 - Positive 2 - Indeterm.		*																														
E1_1_4_2	<input type="text"/> After breastfeeding	1.Date	D D M M Y Y Y Y		*																														
		2.Result	0 - Negative 1 - Positive 2 - Indeterm.		*																														
E1_1_4_3	<input type="text"/> Any PCR	1.Date	D D M M Y Y Y Y		*																														
		2.Result	0 - Negative 1 - Positive 2 - Indeterm.		*																														
E1_1_5	Maternal lifelong ART during pregnancy (started at or before 12w)	0 - No	1 - Yes	-5 Unknown	*																														
E1_1_6	Maternal CD4 in pregnancy	0 - No	1 - Yes	-5 Unknown	*																														
E1_1_6_1	If yes, Absolute count in pregnancy				*																														
E2_1	Is the child known to be HIV+?	0 - No	1 - Yes	-5 Unknown	** If No/ Unkn, go to F_1																														
E2_2	If Yes, date of diagnosis	M M Y Y Y Y		*																															
E2_3	Currently on ART?	0 - No	1 - Yes	-5 Unknown	* If No/Unkn, go to E2_3_2																														
E2_3_1	If yes, adherence	Good	Poor	Uncertain	*																														
E2_3_1_1	ART start date (if ever on ART)	M M Y Y Y Y		*																															
E2_3_2	If no, ever on ART?	0 - No	1 - Yes	-5 Unknown	*																														
E2_3_2_1	ART start date (if ever on ART)	M M Y Y Y Y		*																															
E2_3_2_2	ART last received (if not currently on ART)	M M Y Y Y Y		*																															
E2_3_3	If ever on ART: Regimen	<table border="1"> <tr><td>1</td><td>Abacavir ABC</td><td></td></tr> <tr><td>2</td><td>Stavudine d4T</td><td></td></tr> <tr><td>3</td><td>Zidovudine AZT</td><td></td></tr> <tr><td>4</td><td>Lamivudine 3TC</td><td></td></tr> <tr><td>5</td><td>Didanosine ddl</td><td></td></tr> <tr><td>6</td><td>Kaletra KLT/Alluvia</td><td></td></tr> <tr><td>7</td><td>Efavirenz EFV</td><td></td></tr> <tr><td>8</td><td>Nevirapine NVP</td><td></td></tr> <tr><td>-8</td><td>OTHER</td><td></td></tr> <tr><td colspan="3">SPECIFY</td></tr> </table>			1	Abacavir ABC		2	Stavudine d4T		3	Zidovudine AZT		4	Lamivudine 3TC		5	Didanosine ddl		6	Kaletra KLT/Alluvia		7	Efavirenz EFV		8	Nevirapine NVP		-8	OTHER		SPECIFY			* Mark with More than one can apply
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8	Nevirapine NVP																																		
-8	OTHER																																		
SPECIFY																																			
E2_3_4	First or second line?	First	Second	-4 N/A	**																														

**TREATMENT RESPONSE STUDY  
CRF-B POOLING – CASE REPORT FORM - BASELINE**

E2_3_5_1	CD4 at ART initiation	1	Abs		*					
E2_3_5_2		2	%							
E2_3_5_3	VL at ART initiation	1	VL		*					
E2_3_5_4		2	Log							
E2_6	Latest CD4 (date)	D	D	M	M	Y	Y	Y	Y	*
E2_3_6_1		1	Abs		*					
E2_3_6_2		2	%							
E2_7	Latest VL (date)	D	D	M	M	Y	Y	Y	Y	*
E2_3_7_1		1	VL		*					
E2_3_7_2		2	Log							
E2_8	ARV Clinic				*					
E2_9	WHO clinical stage (current)				**					
F1_1	Current/ recent <b>upper</b> respiratory tract infection (RTI) (rhinorrhoea, blocked nose, ear ache, sneezing, conjunctivitis) <i>Note: recent refers to preceding 2 weeks</i>	0 - No		1 - Yes		**				
F1_2	Current/ recent lower respiratory tract infection (RTI)(recent hospitalisation, radiological evidence, difficulty breathing, high fever)	0 - No		1 - Yes		**				
F1_3	Has child received antibiotics recently (past 2 weeks)?	0 - No		1 - Yes		-5 Unknown		**		
F1_3_1	If yes, how many days?					*				
F1_3_2	Name of antibiotic if known					*				
F1_4	Current/ recent diarrhoeal disease	0 - No		1 - Yes		**				
F1_5	Current/ recent worm infestation	0 - No		1 - Yes		**				
F1_6	Reason for current hospitalization					**				
F1_6_1	Date of hospital admission	D	D	M	M	Y	Y	Y	Y	

**F2 CHRONIC ILLNESS**

F2_1	<b>Known chronic illness?</b> (Excluding HIV, but including HIV-associated chronic conditions such as chronic lung disease, nephropathy etc.)		0 - No		1 - Yes		If No Go to G_1
F2_1_1	Cardiac	Specify	0 - No		1 - Yes		* If No, Go to F2_1_2
F2_1_2	Respiratory	Specify	0 - No		1 - Yes		* If No, Go to F2_1_3
F2_1_3	Gastrointestinal	Specify	0 - No		1 - Yes		* If No, Go to F2_1_4
F2_1_4	Neurological	Specify	0 - No		1 - Yes		* If No, Go to F2_1_5
F2_1_5	Other	Specify	0 - No		1 - Yes		* If No, Go to G_1

**TREATMENT RESPONSE STUDY**  
**CRF-B POOLING – CASE REPORT FORM - BASELINE**

G_1	Is RTHC available?	0 - No	1 - Yes	**	If No, Go to H_1										
G_1_1	Dewormed in the previous 6 months?	0 - No	1 - Yes	*	If No, Go to G_1_2										
G_1_2	Received Vitamin A in the past 6 months?	0 - No	1 - Yes	*											
G_1_3	Poor growth documented?	0 - No	1 - Yes	*											
G_1_3_1	Description of poor growth	<table border="1"> <tr><td>1</td><td>Crossing growth centiles past 3 months / longer (not static)</td></tr> <tr><td>2</td><td>Static growth curve past 3 months or longer</td></tr> <tr><td>3</td><td>Weight loss past 3 months or longer</td></tr> <tr><td>4</td><td>Acute weight loss past month</td></tr> <tr><td>5</td><td>Growing along &lt;-2 Z-line</td></tr> </table>				1	Crossing growth centiles past 3 months / longer (not static)	2	Static growth curve past 3 months or longer	3	Weight loss past 3 months or longer	4	Acute weight loss past month	5	Growing along <-2 Z-line
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3	Weight loss past 3 months or longer														
4	Acute weight loss past month														
5	Growing along <-2 Z-line														
G_1_4	Immunisation schedule up to date?	0 - No	1 - Yes	-5 Unknown	*										

Birth	BCG	OPV(0)				
6 Weeks	OPV(1)	RV(1)	DTaP – IPV/Hib(1)	HepB (1)	PCV <sub>7</sub> (1)	DTP - Hib
10 Weeks	HepB (2)	DTaP – IPV/Hib(2)	DTP - Hib	OPV(2)		
14 Weeks	RV (2)	DTaP – IPV/Hib(3)	HepB (3)	PCV <sub>7</sub> (2)	DTP - Hib	OPV3
9 Months	Measles (1)	PCV <sub>7</sub> (3)				
18 Months	Measles (2)	DTaP – IPV/Hib(4)	DTP	OPV4		
5 Years	DT	OPV5				
6 Years	Td Vaccine					
12 Year	Td Vaccine					

H_1	Does child currently take chronic medication	0 - No	1 - Yes	**	If No, Go to H_2																																				
H_1_1	Specify:	<table border="1"> <tr><td>1</td><td>ART</td><td></td><td></td></tr> <tr><td>2</td><td>IPT</td><td></td><td></td></tr> <tr><td>3</td><td>Bactrim</td><td></td><td></td></tr> <tr><td>4</td><td>Steroid MDI's</td><td></td><td></td></tr> <tr><td>5</td><td>Anticonvulsants</td><td></td><td></td></tr> <tr><td>6</td><td>MVT</td><td></td><td></td></tr> <tr><td>7</td><td>Iron supplements</td><td></td><td></td></tr> <tr><td>-8</td><td>Other</td><td></td><td></td></tr> <tr><td colspan="4">Specify</td></tr> </table>				1	ART			2	IPT			3	Bactrim			4	Steroid MDI's			5	Anticonvulsants			6	MVT			7	Iron supplements			-8	Other			Specify			
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7	Iron supplements																																								
-8	Other																																								
Specify																																									

H_2 Acute medication (tick appropriate)		0 - No	1 - Yes	** If no, go to H_3
Medication Type	Yes/No			
Antibiotics _____				
Antibiotics _____				
Antibiotics _____				
Antibiotics _____				
Steroids				
De-worming				
Vitamin A				
Zinc				

**TREATMENT RESPONSE STUDY**  
**CRF-B POOLING – CASE REPORT FORM - BASELINE**

Asthma agents (Bronchodilators)			
Other- Specify: 1.			
2.			
3.			
4.			

**H\_3 TB Treatment** (if yes to any H\_3\_1 to H\_3\_4, fill in Drug table below)

H_3_1	TB treatment?	0-No		1-Yes				**	
H_3_1_1	When started?	d	d	m	m	y	y	y	*
H_3_2	IPT started?	0-No		1-Yes				**	
H_3_2_1	When started?	d	d	m	m	y	y	y	*
H_3_3	IPT stopped?	0-No		1-Yes				**	
H_3_3_1	If yes, when stopped?								*
H_3_4	MDR/XDR TB prophylaxis started?	0-No		1-Yes				**	
H_3_4_1	When started?	d	d	m	m	y	y	y	*

Drug (for active disease or prophylaxis)	Yes/No	Date started	Dosage
Group 1	Isoniazid		
	Rifampicin		
	Rifabutin		
	Pyrazinamide		
	Ethambutol		
Group 2	Amikacin		
	Streptomycin		
	Kanamycin		
Group 3	Ofloxacin		
	Moxifloxacin		
	Levofloxacin		
Group 4	Ethionamide		
	Terizidone		
	PAS		
Group 5	Linezolid		
	Clarithromycin		
	Cycloserine		
Group 6	Prednisone		
	Dexamethazone		
	Hydrocortisone		
	Other - Specify		

**I CLINICAL EXAMINATION**

**L\_1 NUTRITIONAL PARAMETER**

L_1_1	Weight	<input type="text"/>	Kg	**
L_1_2	Length/Height	<input type="text"/>	Cm	**



**TREATMENT RESPONSE STUDY  
CRF-B POOLING – CASE REPORT FORM - BASELINE**

I\_1\_3 MUAC  Cm \*\*

**I\_2 GENERAL EXAMINATION**

I\_2\_1 Temperature  °C \*\*

I\_2\_1\_1 Site

1	Axillary
2	Oral
3	Rectal
4	Ear
-8	Other
Specify Other	

I\_2\_2 BCG Scar?  0 - No  1 - Yes \*\*

I\_2\_3 Clubbing  0 - No  1 - Yes \*\*

I\_2\_4 Parotid enlargement  0 - No  1 - Yes \*\*

I\_2\_5 Lymph nodes?  0 - No  1 - Yes \*\*

I\_2\_5\_1 Lymph node description  \*\* If No then Go to I\_2\_6

	1. Site	2. Size (cm)	3.Consistency	4.Tender	5.Sinus	6.Fistula	7.Other complication
1	Cervical						
2	Submandibular						
3	Axillary						
4	Inguinal						
5	Eptrochlear						
6	Generalized						

Consistency: soft, firm, hard, matted.

I\_2\_6 Pallor  0 - No  1 - Yes \*\*

I\_2\_7 Dehydration  0 - No  1 - Yes \*\*

I\_2\_8 Hypersensitivity phenomena  0 - No  1 - Yes \*\*

I\_2\_8\_1

1	Erythema induratum	
2	Phlyctens	
3	Tuberculids	
-8	Other	
Specify		

If No, then Go to I\_3\_1

**I\_3 SYSTEM EXAMINATION**

I\_3\_1 Respiratory Rate (10 – 150)  \*\*

I\_3\_2 Difficulty breathing/ increased work of breathing?  0 - No  1 - Yes \*\*

If no, go to I\_3\_3

I\_3\_2\_1 Specify

1	Nasal flare	
2	Grunting	
3	Rib retractions	
4	Supplemental oxygen	

Mark with ✓  
More than one can apply

I\_3\_3 Signs of chronic lung disease  0 - No  1 - Yes \*\*

If No then Go to I\_3\_4

I\_3\_3\_1

1	LIP	
2	Chest deformity	

Mark with ✓

**TREATMENT RESPONSE STUDY**  
**CRF-B POOLING – CASE REPORT FORM - BASELINE**

		3	Coarse crackles			More than one can apply
		4	Pulmonary hypertension			
L_3_4	Wheeze	0 - No		1 - Yes		** If No then Go to L_3_5
	L_3_4_1	1	Monophonic			*
		2	Polyphonic			
L_3_5	Stridor	0 - No		1 - Yes		** If no, go to L_3_6
L_3_6	URTI	0 - No		1 - Yes		** If No then Go to L_3_7
	L_3_6_1 Otorrhoea?	0 - No		1 - Yes		*
	L_3_6_2 If yes, which side?	Left	Right	Both		
L_3_7	LRTI	0 - No		1 - Yes		** If no, go to L_4
	L_3_7_1 If yes	0 - No		1 - Yes		*
	L_3_7_1_1 Crackles/crepitations	1	Bilateral			
	L_3_7_1_1_1 If yes:	2	Unilateral left			
		3	Unilateral right			
	L_3_7_1_2 Hyperinflation	0 - No		1 - Yes		*
	L_3_7_1_2_1 If yes:	1	Bilateral			
		2	Unilateral left			
		3	Unilateral right			
	L_3_7_1_3 Effusion	0 - No		1 - Yes		*
	L_3_7_1_3_1 If yes:	1	Bilateral			
		2	Unilateral left			
		3	Unilateral right			
	L_3_7_1_4 Reduced air entry	0 - No		1 - Yes		*
	L_3_7_1_4_1 If yes:	1	Bilateral			
		2	Unilateral left			
		3	Unilateral right			
	L_3_7_1_5 Other findings	0 - No		1 - Yes		
	L_3_7_1_5_1 If yes, specify					
L_4	<b>ABDOMEN</b>					
L_4_1	Liver enlargement	0 - No		1 - Yes		** If No then Go to L_4_2
	L_4_1_1 Size (give exact size, not a range)					*
L_4_2	Spleen enlargement	0 - No		1 - Yes		** If No then Go to L_5
	L_4_2_1 Size					*
L_5	<b>CNS</b>					
L_5_1	Depressed LOC	0 - No		1 - Yes		** If No then Go to L_5_2
	L_5_1_1 Glasgow coma scale (3-15)					*
L_5_2	Focal signs	0 - No		1 - Yes		**
L_5_3	Meningism	0 - No		1 - Yes		**

**TREATMENT RESPONSE STUDY  
CRF-B POOLING – CASE REPORT FORM - BASELINE**

I_5_4	Raised intracranial pressure	0 - No	1 - Yes	**							
<b>I_6</b>	<b>SPECIAL INVESTIGATION ROUTINE</b>										
I_6_1	Mantoux	0 - No	1 - Yes	**	If No then Go to I_6_2						
I_6_1_1	Date	d	D	m	m	y	y	y	y	*	
I_6_1_2	Size									mm	*
I_6_2	CXR	0 - No	1 - Yes	**	If No, then Go to I_6_3						
I_6_2_1	Date	d	D	m	m	y	y	y	y	*	
I_6_3	HIV	0 - No	1 - Yes	**	If No, then Go to I_6_4						
I_6_3_2	Test Type (choose the most definitive one)	1	PCR	*							
		2	ELISA	*							
		3	Rapid	*							
I_6_3_1	Date	d	D	m	m	y	y	y	y	*	
	Result	1	Positive	*							
		2	Negative	*							
		3	Indeterminate	*							

I_6_4	Blood tests	0 - No	1 - Yes	*
	Test			Results
	Date (dd/mm/yyyy)			
	Hb			
	MCV			
	WCC			
	Neutrophil count			
	Lymphocyte count			
	Platelet count			
	ESR			
	CRP			
	Albumin			
	Procalcitonin			
	CD4 count absolute			
	CD4 count %			
	Viral load (Log value)			
	Viral load (Copies/ml)			
	Other, Specify _____			

**TREATMENT RESPONSE STUDY  
CRF-B POOLING – CASE REPORT FORM - BASELINE**

**J RESULTS**

**J1 BACTERIOLOGY RESULTS**

Sample	Y/N	Date taken	Smear: Neg, Scanty, 1+, 2+, 3+	MGIT: Neg, pos	TTP (Time to pos culture) 1-42 days	Hain: MTB complex, NTM	DST: INH sens or res, RIF sens or res	Xpert: Pos (low, med, hi), Neg, Invalid, error	Xpert Rif Sensitivity: Sens or res or indeterminate
GA1									
GA2									
IS1									
IS2									
Stool 1									
Stool 2									
NPA1									
NPA2									
NSTA1									
NSTA2									

**J2 ADDITIONAL BACTERIOLOGY (GIVE DATE , RESULT)**

Sample	Date Taken	TB Smear	TB Culture	Bacterial Culture	Cell count
Abdominal fluid					
Bone biopsy					
Cerebrospinal fluid					
Ear swab					
Joint aspirate					
Lung tissue					
Pericardial fluid					
Pleural fluid					
Skin					
Urine					
Broncho-alveolar lavage					
Fine needle aspirate Site					
Bone marrow aspirate					
Tracheal aspirate					
Gastric aspirate					
Other					

**J3 VIROLOGY RESULTS**

Sample	Date Taken	Result
NPA		
Urine		
Stool		
Sputum		
Other (specify)		

**J4 ADDITIONAL IMAGING**

J\_4\_1

CT Chest done

0 - No

1 - Yes

\*\*

**TREATMENT RESPONSE STUDY  
CRF-B POOLING – CASE REPORT FORM - BASELINE**

J_4_1_1	Date	d	D	m	m	y	y	y	y	*	If No/ Unknown then Go to J_4_2	
J_4_1_2	Lymphadenopathy	0 - No		1 - Yes						*		
J_4_1_3	Ring enhancement	0 - No		1 - Yes						*		
J_4_1_4	Other findings	Specify									*	
J_4_1_5	Diagnosis normal?	0 - No		1 - Yes						*		
J_4_1_6	If Abnormal	1	Certain TB		2	Possible TB				*		
		3	Normal		4	Not TB				*		
J_4_2	CT Brain done	0 - No		1 - Yes						**	If No/ Unknown then Go to J_4_3	
J_4_2_1	Date	D	D	m	m	y	y	y	y	*		
J_4_2_2	Basal meningeal enhancement	0 - No		1 - Yes						*		
J_4_2_3	Hydrocephalus	0 - No		1 - Yes						*		
J_4_2_4	Tuberculoma	0 - No		1 - Yes						*		
J_4_2_5	Infarction	0 - No		1 - Yes						*		
J_4_2_6	Other findings	Specify									*	
J_4_2_7	Diagnosis normal?	0 - No		1 - Yes						*		
J_4_2_8	If Abnormal	1	Certain TB		2	Possible TB				*		
		3	Normal		4	Not TB				*		
J_4_3	MRI Brain done	0 - No		1 - Yes						**	If No/ Unknown then Go to J_4_4	
J_4_3_1	Date	D	D	m	m	y	y	y	y	*		
J_4_3_2	Basal meningeal enhancement	0 - No		1 - Yes						*		
J_4_3_3	Hydrocephalus	0 - No		1 - Yes						*		
J_4_3_4	Tuberculoma	0 - No		1 - Yes						*		
J_4_3_5	Infarction	0 - No		1 - Yes						*		
J_4_3_6	Other findings	Specify									*	
J_4_3_7	Diagnosis normal?	0 - No		1 - Yes						*		
J_4_3_8	If Abnormal	1	Certain TB		2	Possible TB				*		
		3	Normal		4	Not TB				*		
J_4_4	Air Encephalogram done	0 - No		1 - Yes						**	If No/ Unknown then Go to J_4_5	
J_4_4_1	Date	D	D	m	m	y	y	y	y	*		
J_4_4_2	Communicating Hydrocephalus	0 - No		1 - Yes						*		
J_4_5	Abdominal Ultrasound done	0 - No		1 - Yes						**	If No/ Unknown then Go to J_4_6	
J_4_5_1	Date	D	D	m	m	y	y	y	y	*		
J_4_5_2	Lymphadenopathy	0 - No		1 - Yes						*		
J_4_5_3	Hepatomegaly	0 - No		1 - Yes						*		
J_4_5_4	Splenomegaly	0 - No		1 - Yes						*		
J_4_5_5	Other findings	Specify									*	
J_4_5_6	Diagnosis normal?	0 - No		1 - Yes						*		
J_4_5_7	If Abnormal	1	Compatible with TB		2	Not TB				*		
		3	Normal							*		
J_4_6	Bronchoscopy done	0 - No		1 - Yes						**	If No/ Unknown then Go to J_4_7	
J_4_6_1	Date	d	D	m	m	y	y	y	y	*		
J_4_6_2	Airway compression	0 - No		1 - Yes						*		
J_4_6_3	Trachea	0 - No		1 - Yes						*		



**TREATMENT RESPONSE STUDY  
CRF-B POOLING – CASE REPORT FORM - BASELINE**

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**TR: POOLING STUDY**  
**CRF-FU – CASE REPORT FORM – FOLLOW-UP**

DATE	D	D	M	M	Y	Y	Y	Y
TIME			H					



\*\* Compulsory question  
 \* Compulsory question depending on parent question  
 Make use of jump instructions  
 Make use of a black pen only

**A. HOUSEHOLD**

A_1	Has there been a change in primary caregiver?	0-no	1-yes	**	If no, go to B																	
If yes:																						
A_1_1	Who is primary caregiver (tick one)	<table border="1"> <tr><td><input type="checkbox"/></td><td>Mother</td></tr> <tr><td><input type="checkbox"/></td><td>Father</td></tr> <tr><td><input type="checkbox"/></td><td>Grandparent</td></tr> <tr><td><input type="checkbox"/></td><td>Other family</td></tr> <tr><td><input type="checkbox"/></td><td>Other community member, not fostering</td></tr> <tr><td><input type="checkbox"/></td><td>Place of safety (children's home)</td></tr> <tr><td><input type="checkbox"/></td><td>Foster care</td></tr> <tr><td><input type="checkbox"/></td><td>Adoptive parent</td></tr> </table>				<input type="checkbox"/>	Mother	<input type="checkbox"/>	Father	<input type="checkbox"/>	Grandparent	<input type="checkbox"/>	Other family	<input type="checkbox"/>	Other community member, not fostering	<input type="checkbox"/>	Place of safety (children's home)	<input type="checkbox"/>	Foster care	<input type="checkbox"/>	Adoptive parent	*
<input type="checkbox"/>	Mother																					
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<input type="checkbox"/>	Place of safety (children's home)																					
<input type="checkbox"/>	Foster care																					
<input type="checkbox"/>	Adoptive parent																					

**B SYMPTOMS**

B_1	Cough	0 - No	1 - Yes	-5 - Unknown	**	If Yes, go to B_1_1 If No, go to B_1_2																								
B_1_1 If Yes:																														
B_1_1_1	Cough pattern	<table border="1"> <tr><td>1</td><td>Acute</td></tr> <tr><td>2</td><td>Prolonged</td></tr> <tr><td>3</td><td>Recurrent</td></tr> </table>				1	Acute	2	Prolonged	3	Recurrent	*																		
1	Acute																													
2	Prolonged																													
3	Recurrent																													
B_1_1_1_1	If acute, duration	<table border="1"> <tr><td></td><td>Days</td></tr> </table>					Days	*																						
	Days																													
B_1_1_2	Is cough associated with	<table border="1"> <tr><td>0 - No</td><td>1 - Yes</td><td>-5 - Unknown</td></tr> <tr><td>0 - No</td><td>1 - Yes</td><td>-5 - Unknown</td></tr> <tr><td>0 - No</td><td>1 - Yes</td><td>-5 - Unknown</td></tr> <tr><td>0 - No</td><td>1 - Yes</td><td>-5 - Unknown</td></tr> <tr><td>0 - No</td><td>1 - Yes</td><td>-5 - Unknown</td></tr> <tr><td>0 - No</td><td>1 - Yes</td><td>-5 - Unknown</td></tr> <tr><td>0 - No</td><td>1 - Yes</td><td>-5 - Unknown</td></tr> <tr><td>0 - No</td><td>1 - Yes</td><td>-5 - Unknown</td></tr> </table>				0 - No	1 - Yes	-5 - Unknown	0 - No	1 - Yes	-5 - Unknown	0 - No	1 - Yes	-5 - Unknown	0 - No	1 - Yes	-5 - Unknown	0 - No	1 - Yes	-5 - Unknown	0 - No	1 - Yes	-5 - Unknown	0 - No	1 - Yes	-5 - Unknown	0 - No	1 - Yes	-5 - Unknown	*
0 - No	1 - Yes	-5 - Unknown																												
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Exertion																														
Feeding																														
Excitement																														
Allergens (e.g. smoke/dust)																														
Night-time																														
Upper respiratory tract infections																														
Other																														
Specify:																														
B_1_1_3	Day to day variation	<table border="1"> <tr><td>1</td><td>Every day</td></tr> <tr><td>2</td><td>Every second day</td></tr> <tr><td>3</td><td>Occasionally</td></tr> </table>				1	Every day	2	Every second day	3	Occasionally	*																		
1	Every day																													
2	Every second day																													
3	Occasionally																													
B_1_1_4	Character	<table border="1"> <tr><td>1</td><td>Mostly wet</td></tr> <tr><td>2</td><td>Mostly dry</td></tr> </table>				1	Mostly wet	2	Mostly dry	*																				
1	Mostly wet																													
2	Mostly dry																													



**TR: POOLING STUDY**  
**CRF-FU – CASE REPORT FORM – FOLLOW-UP**

B_2	Wheezing	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1 - Yes</td> <td style="width: 33%;">-5 - Unknown</td> </tr> </table>	0 - No	1 - Yes	-5 - Unknown	** If No, go to B_3					
0 - No	1 - Yes	-5 - Unknown									
B_2_1	If yes, duration of wheezing	days	*								
B_2_2	Pattern of wheezing	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">1.Acute (&lt;14d)</td> <td style="width: 33%;">2.Prolonged (≥14d)</td> <td style="width: 33%;">3.Recurrent</td> </tr> </table>	1.Acute (<14d)	2.Prolonged (≥14d)	3.Recurrent	*					
1.Acute (<14d)	2.Prolonged (≥14d)	3.Recurrent									
B_2_2_1	If prolonged or recurrent, is wheeze associated with:										
B_2_2_1_1	Exertion	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1 - Yes</td> <td style="width: 33%;">-5 - Unknown</td> </tr> </table>	0 - No	1 - Yes	-5 - Unknown	*					
0 - No	1 - Yes	-5 - Unknown									
B_2_2_1_2	Feeding	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1 - Yes</td> <td style="width: 33%;">-5 - Unknown</td> </tr> </table>	0 - No	1 - Yes	-5 - Unknown	*					
0 - No	1 - Yes	-5 - Unknown									
B_2_2_1_3	Excitement	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1 - Yes</td> <td style="width: 33%;">-5 - Unknown</td> </tr> </table>	0 - No	1 - Yes	-5 - Unknown	*					
0 - No	1 - Yes	-5 - Unknown									
B_2_2_1_4	Allergens (e.g. smoke/dust)	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1 - Yes</td> <td style="width: 33%;">-5 - Unknown</td> </tr> </table>	0 - No	1 - Yes	-5 - Unknown	*					
0 - No	1 - Yes	-5 - Unknown									
B_2_2_1_5	Night-time	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1 - Yes</td> <td style="width: 33%;">-5 - Unknown</td> </tr> </table>	0 - No	1 - Yes	-5 - Unknown	*					
0 - No	1 - Yes	-5 - Unknown									
B_2_2_1_6	Upper respiratory tract infections	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1 - Yes</td> <td style="width: 33%;">-5 - Unknown</td> </tr> </table>	0 - No	1 - Yes	-5 - Unknown	*					
0 - No	1 - Yes	-5 - Unknown									
B_2_2_1_7	Other	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1 - Yes</td> <td style="width: 33%;">-5 - Unknown</td> </tr> </table>	0 - No	1 - Yes	-5 - Unknown	*					
0 - No	1 - Yes	-5 - Unknown									
	B_2_2_1_7_1 If other, specify		*								
B_3	Fever	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1 - Yes</td> <td style="width: 33%;">-5 - Unknown</td> </tr> </table>	0 - No	1 - Yes	-5 - Unknown	** If No, go to B_4					
0 - No	1 - Yes	-5 - Unknown									
	If Yes										
B_3_1	Fever Condition	<table border="1" style="display: inline-table;"> <tr> <td style="width: 20%;">1</td> <td>New</td> </tr> <tr> <td>2</td> <td>Worse</td> </tr> <tr> <td>3</td> <td>Persistent</td> </tr> <tr> <td>4</td> <td>Improved</td> </tr> </table>	1	New	2	Worse	3	Persistent	4	Improved	*
1	New										
2	Worse										
3	Persistent										
4	Improved										
	B_3_1_2 If new, duration:		*								
	1 Before hospital admission	Days									
	2 Since hospital admission	Days									
B_3_2	Diurnal variation	<table border="1" style="display: inline-table;"> <tr> <td style="width: 20%;">1</td> <td>Daily</td> </tr> <tr> <td>2</td> <td>Intermittent</td> </tr> </table>	1	Daily	2	Intermittent	*				
1	Daily										
2	Intermittent										
B_3_3	Highest recorded temperature if available (-5 if NA)		*								
B_4	Lack of appetite	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1 - Yes</td> <td style="width: 33%;">-5 - Unknown</td> </tr> </table>	0 - No	1 - Yes	-5 - Unknown	** If no, go to B_5					
0 - No	1 - Yes	-5 - Unknown									
B_4_1	Lack of appetite for solids?	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1- Yes</td> <td style="width: 33%;">N/A</td> </tr> </table>	0 - No	1- Yes	N/A	*					
0 - No	1- Yes	N/A									
B_4_2	Lack of appetite for liquids	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1- Yes</td> </tr> </table>	0 - No	1- Yes	*						
0 - No	1- Yes										
B_5	Diminished playfulness	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1 - Yes</td> <td style="width: 33%;">-5 - Unknown</td> </tr> </table>	0 - No	1 - Yes	-5 - Unknown	** If no, go to B_6					
0 - No	1 - Yes	-5 - Unknown									
B_6	Lethargy (sleepiness)	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1 - Yes</td> <td style="width: 33%;">-5 - Unknown</td> </tr> </table>	0 - No	1 - Yes	-5 - Unknown	** If no, go to B_7					
0 - No	1 - Yes	-5 - Unknown									
B_6_1	Awake in the day but not engaging in usual activities	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1 - Yes</td> <td style="width: 33%;">-5 - Unknown</td> </tr> </table>	0 - No	1 - Yes	-5 - Unknown	*					
0 - No	1 - Yes	-5 - Unknown									
B_6_2	Mostly asleep / lying down	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1 - Yes</td> <td style="width: 33%;">-5 - Unknown</td> </tr> </table>	0 - No	1 - Yes	-5 - Unknown	*					
0 - No	1 - Yes	-5 - Unknown									
B_7	Other symptoms?	<table border="1" style="display: inline-table;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1 - Yes</td> </tr> </table>	0 - No	1 - Yes	** If no, go to C_1						
0 - No	1 - Yes										
B_7_1	If yes, specify	<table border="1" style="display: inline-table;"> <thead> <tr> <th style="width: 70%;">Symptom</th> <th>Duration (d)</th> </tr> </thead> <tbody> <tr> <td> </td> <td> </td> </tr> <tr> <td> </td> <td> </td> </tr> <tr> <td> </td> <td> </td> </tr> </tbody> </table>	Symptom	Duration (d)							
Symptom	Duration (d)										

**TR: POOLING STUDY**  
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**C TB SOURCE CASE IDENTIFICATION**

C_1	Was a TB source case identified at baseline?	Yes	No	**	If No go to D_1
C_2	Is there ongoing contact with any of the identified source cases?	0 - No	1 - Yes	*	
C_3	Has the source case completed treatment?	0 - No	1 - Yes	-5 Unknown	*

**D NEW TB EXPOSURE**

D_1	Has the child had any contact with a <b>NEW</b> Confirmed or Suspected TB index case since last seen?	0 - No	1 - Yes	-5 Unknown	**	If No/Unk, then Go to E_1				
D_1_1	If yes, how many TB contacts? <i>Complete a TB contact form for each</i>	1 One	2 Two	3 Three	4 Four or more	*				
D_1_2	Did the child start IPT for this contact episode?	0 - No	1 - Yes	-5 Unknown	*					
D_1_2_1	If Yes, at which clinic/hospital						*			
D_1_2_2	If Yes, when did the child start on IPT?	D	D	M	M	Y	Y	Y	Y	*

**E HIV POSITIVE CHILDREN**

E1	Currently on ART?	0 - No	1 - Yes	-5 Unknown	**	If No/Unkn, then Go to E2_2							
E2	1 If yes, adherence	Good	Poor	Uncertain	*								
2	Was ART started since previous visit?	0 - No	1 - Yes	*									
2_1	If yes, date of starting ART	D	D	M	M	Y	Y	Y	Y	*			
3	Was ART stopped since previous visit?	0 - No	1 - Yes	*									
3_1	If yes, date of stopping ART:	D	D	M	M	Y	Y	Y	Y	*			
4	Was ART changed since previous visit?	0 - No	1 - Yes	*									
4_1	If yes, date of changing ART:	D	D	M	M	Y	Y	Y	Y	*			
5	If on ART: Regimen	1 Abacavir	2 Stavudine	3 Zidovudine	4 Lamivudine	5 Didanosine	6 Kaletra	7 Efavirenz	8 Nevirapine	-8 OTHER	SPECIFY	*	Mark with ✓ More than one can apply
E3	ARV Clinic											*	

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E4 WHO clinical stage (current)

**F CONCURRENT ILLNESS**

F\_1 Current/ recent upper respiratory tract infection (RTI) (rhinorrhoea, blocked nose, ear ache, sneezing, conjunctivitis)  
*Note: recent refers to preceding 2 weeks*

0 - No  1 - Yes \*\* If No, Go to F\_2

F\_2 Current/ recent lower respiratory tract infection (RTI)(recent hospitalisation, radiological evidence, difficulty breathing, high fever)

0 - No  1 - Yes \*\* If No, Go to F\_3

F\_3 Has child received antibiotics recently (past 2 weeks)?  
 If yes, how many days?  
 Name of antibiotic if known

0 - No  1 - Yes  -5 Unknown \*\* If No, go to F\_4

F\_4 Current/ recent diarrhoeal disease

0 - No  1 - Yes \*\* If No, Go to F\_5

F\_5 Current/ recent worm infestation

0 - No  1 - Yes \*\* If No, Go to F\_6

F\_6 Recent deworming?  
 F\_6\_1 If yes, Date of deworming

0 - No  1 - Yes \*\* If No, Go to F\_7

D  D  M  M  Y  Y  Y  Y

F\_7 Hospitalised?  
 F\_7\_1 If yes, reason for hospitalization  
 F\_7\_2 Date of admission

0 - No  1 - Yes \*\* If No, Go to G\_1

D  D  M  M  Y  Y  Y  Y

G\_1 Does child currently take chronic medication

0 - No  1 - Yes \*\* If No, Go to G\_2

G\_1\_1 Specify:

1	ART	
2	IPT	
3	Bactrim	
4	Inhaled steroids	
5	Anticonvulsants	
6	MVT	
7	Iron supplements	
-8	Other	
Specify		

G\_2 Acute medication?

Yes  No \*\* If No, go to G\_3

**G\_2\_1 Acute medication (tick appropriate)**

Medication Type	Yes/No	Days of treatment	IV (1) Oral (2) Both (3) Nebulisation (4)
Antibiotics-type			
Antibiotics-type			
Antipyretics			
Anti-inflammatories			
Steroids-type			
Herbal/Traditional			
De-worming			
Vitamin A			
Zinc			
Asthma agents			

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<b>(Bronchodilators)</b>																			
<b>Other- Specify</b>																			
1																			
2																			
3																			
4																			
<b>G_3 TB Treatment OR prophylaxis (If YES to any of the following, complete G_3_7)</b>			<b>0-No</b> <b>1-Yes</b> <b>** If no, go to H_1</b>																
G_3_1	TB treatment?	<table border="1"> <tr> <td colspan="4"><b>0-No</b></td> <td colspan="4"><b>1-Yes</b></td> </tr> <tr> <td>D</td><td>D</td><td>M</td><td>M</td> <td>Y</td><td>Y</td><td>Y</td><td>Y</td> </tr> </table>		<b>0-No</b>				<b>1-Yes</b>				D	D	M	M	Y	Y	Y	Y
<b>0-No</b>				<b>1-Yes</b>															
D	D	M	M	Y	Y	Y	Y												
G_3_1_1	When started?	<table border="1"> <tr> <td colspan="4"><b>0-No</b></td> <td colspan="4"><b>1-Yes</b></td> </tr> <tr> <td>D</td><td>D</td><td>M</td><td>M</td> <td>Y</td><td>Y</td><td>Y</td><td>Y</td> </tr> </table>		<b>0-No</b>				<b>1-Yes</b>				D	D	M	M	Y	Y	Y	Y
<b>0-No</b>				<b>1-Yes</b>															
D	D	M	M	Y	Y	Y	Y												
G_3_2	TB treatment stopped?	<table border="1"> <tr> <td colspan="4"><b>0-No</b></td> <td colspan="4"><b>1-Yes</b></td> </tr> <tr> <td>D</td><td>D</td><td>M</td><td>M</td> <td>Y</td><td>Y</td><td>Y</td><td>Y</td> </tr> </table>		<b>0-No</b>				<b>1-Yes</b>				D	D	M	M	Y	Y	Y	Y
<b>0-No</b>				<b>1-Yes</b>															
D	D	M	M	Y	Y	Y	Y												
G_3_2_1	When stopped?	<table border="1"> <tr> <td colspan="4"><b>0-No</b></td> <td colspan="4"><b>1-Yes</b></td> </tr> <tr> <td>D</td><td>D</td><td>M</td><td>M</td> <td>Y</td><td>Y</td><td>Y</td><td>Y</td> </tr> </table>		<b>0-No</b>				<b>1-Yes</b>				D	D	M	M	Y	Y	Y	Y
<b>0-No</b>				<b>1-Yes</b>															
D	D	M	M	Y	Y	Y	Y												
G_3_3	IPT started?	<table border="1"> <tr> <td colspan="4"><b>0-No</b></td> <td colspan="4"><b>1-Yes</b></td> </tr> <tr> <td>D</td><td>D</td><td>M</td><td>M</td> <td>Y</td><td>Y</td><td>Y</td><td>Y</td> </tr> </table>		<b>0-No</b>				<b>1-Yes</b>				D	D	M	M	Y	Y	Y	Y
<b>0-No</b>				<b>1-Yes</b>															
D	D	M	M	Y	Y	Y	Y												
G_3_3_1	When started?	<table border="1"> <tr> <td colspan="4"><b>0-No</b></td> <td colspan="4"><b>1-Yes</b></td> </tr> <tr> <td>D</td><td>D</td><td>M</td><td>M</td> <td>Y</td><td>Y</td><td>Y</td><td>Y</td> </tr> </table>		<b>0-No</b>				<b>1-Yes</b>				D	D	M	M	Y	Y	Y	Y
<b>0-No</b>				<b>1-Yes</b>															
D	D	M	M	Y	Y	Y	Y												
G_3_4	IPT stopped?	<table border="1"> <tr> <td colspan="4"><b>0-No</b></td> <td colspan="4"><b>1-Yes</b></td> </tr> <tr> <td>D</td><td>D</td><td>M</td><td>M</td> <td>Y</td><td>Y</td><td>Y</td><td>Y</td> </tr> </table>		<b>0-No</b>				<b>1-Yes</b>				D	D	M	M	Y	Y	Y	Y
<b>0-No</b>				<b>1-Yes</b>															
D	D	M	M	Y	Y	Y	Y												
G_3_4_1	If yes, when stopped?	<table border="1"> <tr> <td colspan="4"><b>0-No</b></td> <td colspan="4"><b>1-Yes</b></td> </tr> <tr> <td>D</td><td>D</td><td>M</td><td>M</td> <td>Y</td><td>Y</td><td>Y</td><td>Y</td> </tr> </table>		<b>0-No</b>				<b>1-Yes</b>				D	D	M	M	Y	Y	Y	Y
<b>0-No</b>				<b>1-Yes</b>															
D	D	M	M	Y	Y	Y	Y												
G_3_5	MDR/XDR TB prophylaxis started?	<table border="1"> <tr> <td colspan="4"><b>0-No</b></td> <td colspan="4"><b>1-Yes</b></td> </tr> <tr> <td>D</td><td>D</td><td>M</td><td>M</td> <td>Y</td><td>Y</td><td>Y</td><td>Y</td> </tr> </table>		<b>0-No</b>				<b>1-Yes</b>				D	D	M	M	Y	Y	Y	Y
<b>0-No</b>				<b>1-Yes</b>															
D	D	M	M	Y	Y	Y	Y												
G_3_5_1	When started?	<table border="1"> <tr> <td colspan="4"><b>0-No</b></td> <td colspan="4"><b>1-Yes</b></td> </tr> <tr> <td>D</td><td>D</td><td>M</td><td>M</td> <td>Y</td><td>Y</td><td>Y</td><td>Y</td> </tr> </table>		<b>0-No</b>				<b>1-Yes</b>				D	D	M	M	Y	Y	Y	Y
<b>0-No</b>				<b>1-Yes</b>															
D	D	M	M	Y	Y	Y	Y												
G_3_6	MDR/XDR TB prophylaxis stopped?	<table border="1"> <tr> <td colspan="4"><b>0-No</b></td> <td colspan="4"><b>1-Yes</b></td> </tr> <tr> <td>D</td><td>D</td><td>M</td><td>M</td> <td>Y</td><td>Y</td><td>Y</td><td>Y</td> </tr> </table>		<b>0-No</b>				<b>1-Yes</b>				D	D	M	M	Y	Y	Y	Y
<b>0-No</b>				<b>1-Yes</b>															
D	D	M	M	Y	Y	Y	Y												
G_3_6_1	If yes, when stopped?	<table border="1"> <tr> <td colspan="4"><b>0-No</b></td> <td colspan="4"><b>1-Yes</b></td> </tr> <tr> <td>D</td><td>D</td><td>M</td><td>M</td> <td>Y</td><td>Y</td><td>Y</td><td>Y</td> </tr> </table>		<b>0-No</b>				<b>1-Yes</b>				D	D	M	M	Y	Y	Y	Y
<b>0-No</b>				<b>1-Yes</b>															
D	D	M	M	Y	Y	Y	Y												
<b>G_3_7 Drug(for active disease or prophylaxis)    Yes/No    Date started    Dosage</b>																			
<b>Group 1</b>	Isoniazid																		
	Rifampicin																		
	Rifabutin																		
	Pyrazinamide																		
	Ethambutol																		
<b>Group 2</b>	Amikacin																		
	Streptomycin																		
	Kanamycin																		
<b>Group 3</b>	Ofloxacin																		
	Moxifloxacin																		
	Levofloxacin																		
<b>Group 4</b>	Ethionamide																		
	Terizidone																		
	PAS																		
<b>Group 5</b>	Linezolid																		
	Clarithromycin																		
	Cycloserine																		
<b>Group 6</b>	Prednisone																		
	Dexamethazone																		
	Hydrocortisone																		
	<b>Other - Specify</b>																		

**TR: POOLING STUDY**  
**CRF-FU – CASE REPORT FORM – FOLLOW-UP**

**H CLINICAL EXAMINATION**

**H\_1 NUTRITIONAL PARAMETERS**

H_1_1	Weight	<input type="text"/>	kg	**
H_1_2	Length/Height	<input type="text"/>	cm	**
H_1_3	MUAC	<input type="text"/>	cm	**

**H\_2 GENERAL EXAMINATION**

H_2_1	Temperature	<input type="text"/>	**
H_2_2	Clubbing	<input type="text" value="0 - No"/> <input type="text" value="1 - Yes"/>	**
H_2_3	Lymph nodes?	<input type="text" value="0 - No"/> <input type="text" value="1 - Yes"/>	** If No, go to H_2_4

	1. Site	2. Size (cm)	3. Consistency	4. Tender	5. Sinus	6. Fistula	7. Other complication
1	Cervical						
2	Submandibular						
3	Axillary						
4	Inguinal						
5	Epirochlear						
6	Generalized						

*Consistency: soft, firm, hard, matted.*

H_2_4	Pallor	<input type="text" value="0 - No"/> <input type="text" value="1 - Yes"/>	**															
H_2_5	Dehydration	<input type="text" value="0 - No"/> <input type="text" value="1 - Yes"/>	**															
H_2_6	Hypersensitivity phenomena	<input type="text" value="0 - No"/> <input type="text" value="1 - Yes"/>	** If No, go to H_3															
H_2_6_1		<table border="1"> <tr> <td>1</td> <td>Erythema induratum</td> <td></td> </tr> <tr> <td>2</td> <td>Phlyctens</td> <td></td> </tr> <tr> <td>3</td> <td>Tubercuoids</td> <td></td> </tr> <tr> <td>-8</td> <td>Other</td> <td></td> </tr> <tr> <td colspan="3">Specify</td> </tr> </table>	1	Erythema induratum		2	Phlyctens		3	Tubercuoids		-8	Other		Specify			*
1	Erythema induratum																	
2	Phlyctens																	
3	Tubercuoids																	
-8	Other																	
Specify																		

**H\_3 SYSTEM EXAMINATION**

H_3_1	Respiratory Rate (10 -150)	<input type="text"/>	**												
H_3_2	Difficulty breathing/ increased work of breathing?	<input type="text" value="0 - No"/> <input type="text" value="1 - Yes"/>	** If No, go to H_3_3												
H_3_2_1	Specify	<table border="1"> <tr> <td>1</td> <td>Nasal flare</td> <td></td> </tr> <tr> <td>2</td> <td>Grunting</td> <td></td> </tr> <tr> <td>3</td> <td>Rib retractions</td> <td></td> </tr> <tr> <td>4</td> <td>Supplemental oxygen</td> <td></td> </tr> </table>	1	Nasal flare		2	Grunting		3	Rib retractions		4	Supplemental oxygen		** Mark with ✓ More than one can apply
1	Nasal flare														
2	Grunting														
3	Rib retractions														
4	Supplemental oxygen														
H_3_3	Signs of chronic lung disease	<input type="text" value="0 - No"/> <input type="text" value="1 - Yes"/>	** If No, go to H_3_4												
H_3_3_1	If yes:	<table border="1"> <tr> <td>1</td> <td>LIP</td> <td></td> </tr> <tr> <td>2</td> <td>Chest deformity</td> <td></td> </tr> </table>	1	LIP		2	Chest deformity		* Mark with ✓ More than						
1	LIP														
2	Chest deformity														

**TR: POOLING STUDY**  
**CRF-FU – CASE REPORT FORM – FOLLOW-UP**

		3	Coarse crackles			one can apply
		4	Pulmonary hypertension			
H_3_4	Wheeze	0 - No		1 - Yes		** If No, go to H_3_5
	H_3_4_1	1	Monophonic			*
		2	Polyphonic			
H_3_5	Stridor	0 - No		1 - Yes		** If no, go to H_3_6
H_3_6	URTI	0 - No		1 - Yes		** If no, go to H_3_7
	H_3_6_1	0 - No		1 - Yes		*
	H_3_6_2	Left	Right	Both		*
H_3_7	Signs of lower respiratory tract pathology	0 - No		1 - Yes		** If no, go to H_4
	If yes,	0 - No		1 - Yes		
	1 Crackles/crepitations	1	Left			
	1_1 If yes:	2	Right			
		3	Bilat			
	2 Hyperinflation	0 - No		1 - Yes		
	2_1 If yes:	1	Left			
		2	Right			
		3	Bilat			
	3 Effusion	0 - No		1 - Yes		
	3_1 If yes:	1	Left			
		2	Right			
		3	Bilat			
	4 Reduced air entry	0 - No		1 - Yes		
	4_1 If yes:	1	Left			
		2	Right			
		3	Bilat			
	5 Other findings	0 - No		1 - Yes		
	5_1 If yes, specify					
H_4	<b>ABDOMEN</b>					
H_4_1	Liver enlargement	0 - No		1 - Yes		** If No, go to H_4_2
	H_4_1_1	Size (cm) (below costal margin)				*
H_4_2	Spleen enlargement	0 - No		1 - Yes		** If No, go to H_5
	H_4_2_1	Size (cm)				*
H_5	<b>CNS</b>					
H_5_1	Depressed LOC	0 - No		1 - Yes		** If No, go to H_5_2
	H_5_1_1	Glasgow coma scale (3-15)				*
H_5_2	Focal signs	0 - No		1 - Yes		**
H_5_3	Meningism	0 - No		1 - Yes		**

**TR: POOLING STUDY**  
**CRF-FU – CASE REPORT FORM – FOLLOW-UP**

H\_5\_4 Raised intracranial pressure 

0 - No	1 - Yes
--------	---------

 \*\*

**H\_6 OTHER NOTABLE PHYSICAL SIGNS**

H\_6\_1 Other physical signs? 

0 - No	1 - Yes
--------	---------

 \*\* If no, go to L\_1

H\_6\_1\_1 If yes, specify

1	
2	
3	

L\_1 Mantoux 

0 - No	1 - Yes
--------	---------

 \*\*

L\_1\_1 Date 

d	d	m	m	y	y	y	y
---	---	---	---	---	---	---	---

 \* If No, go to L\_2

L\_1\_2 Size 

							mm
--	--	--	--	--	--	--	----

 \*

L\_2 CXR 

0 - No	1 - Yes
--------	---------

 \*\* If No, go to L\_3

L\_2\_1 Date 

d	d	m	m	y	y	y	y
---	---	---	---	---	---	---	---

L\_3 HIV 

0 - No	1 - Yes
--------	---------

 \*\* If No, go to L\_4

L\_3\_1 Test Type(choose the most definitive one)

1	PCR
2	ELISA
3	Rapid

L\_3\_2 Date 

d	d	m	m	y	y	y	y
---	---	---	---	---	---	---	---

 \*

Result

1	Positive
2	Negative
3	Indeterminate

 \*

L_4	Blood tests	YES	NO	**
	Test	Date (dd/mm/yyyy)		Results
	Hb			
	MCV			
	WCC			
	Platelet count			
	Neutrophil count			
	Lymphocyte count			
	ESR			
	CRP			
	Procalcitonin			
	Albumin			
	CD4 count absolute			
	CD4 count %			
	Viral load (Log value)			
	Viral load (Copies/ml)			
	Other, Specify _____			

**TR: POOLING STUDY**  
**CRF-FU – CASE REPORT FORM – FOLLOW-UP**

I_5 BACTERIOLOGY									
Sample	Y / N	Date taken	Smear: Neg, Scanty, 1+, 2+, 3+	MGIT: Neg, pos, contam	TTP (Time to pos culture) 1-42 days	Hain: MTB complex, NTM	DST: INH sens or res, RIF sens or res	Xpert: Pos (low, med, hi), Neg, invalid, error	Xpert Rif Sensitivity: Sens, res, indeterminate
GA									
NPA									
IS									
Spot Sputum									
Fasting sputum									
Stool									
Other, specify									
1									
2									

J ADDITIONAL IMAGING											
J_1	J_1_1	CT Chest done	0 - No				1 - Yes				** If No Go to J_2
	J_1_2	Date	d	d	m	m	y	y	y	y	
	J_1_3	Lymphadenopathy	0 - No				1 - Yes				
	J_1_4	Ring enhancement	0 - No				1 - Yes				
	J_1_5	Other findings	Specify								
	J_1_6	Diagnosis normal?	0 - No				1 - Yes				
	J_1_6	If Abnormal	1	Certain TB			2	Possible TB			
	J_1_6	If Abnormal	3	Normal			4	Not TB			
J_2	J_2_1	CT Brain done	0 - No				1 - Yes				** If No Go to J_3
	J_2_2	Date	d	d	m	m	y	y	y	y	
	J_2_3	Basal meningeal enhancement	0 - No				1 - Yes				
	J_2_4	Hydrocephalus	0 - No				1 - Yes				
	J_2_5	Tuberculoma	0 - No				1 - Yes				
	J_2_6	Infarction	0 - No				1 - Yes				
	J_2_7	Other findings	Specify								
	J_2_8	Diagnosis normal?	0 - No				1 - Yes				
J_2_8	If Abnormal	1	Certain TB			2	Possible TB				
J_2_8	If Abnormal	3	Normal			4	Not TB				
J_3	J_3_1	MRI Brain done	0 - No				1 - Yes				** If No Go to J_4
	J_3_2	Date	d	d	m	m	y	y	y	y	
	J_3_3	Basal meningeal enhancement	0 - No				1 - Yes				
	J_3_4	Hydrocephalus	0 - No				1 - Yes				
	J_3_5	Tuberculoma	0 - No				1 - Yes				
	J_3_6	Infarction	0 - No				1 - Yes				
	J_3_7	Other findings	Specify								
	J_3_8	Diagnosis normal?	0 - No				1 - Yes				
J_3_8	If Abnormal	1	Certain TB			2	Possible TB				
J_3_8	If Abnormal	3	Normal			4	Not TB				
J_4	J_4_1	Air Encephalogram done	0 - No				1 - Yes				** If No Go to J_5
	J_4_2	Date	d	d	m	m	y	y	y	y	
J_4	J_4_2	Communicating Hydrocephalus	0 - No				1 - Yes				
J_5	J_5_1	Abdominal Ultrasound done	0 - No				1 - Yes				** If No Go to J_6
	J_5_2	Date	d	d	m	m	y	y	y	y	
	J_5_3	Lymphadenopathy	0 - No				1 - Yes				
	J_5_4	Hepatomegaly	0 - No				1 - Yes				
	J_5_5	Splenomegaly	0 - No				1 - Yes				
	J_5_6	Other findings	Specify								
	J_5_7	Diagnosis normal?	0 - No				1 - Yes				
	J_5_7	If Abnormal	1	Certain TB			2	Possible TB			
J_5_7	If Abnormal	3	Normal			4	Not TB				



**TR: POOLING STUDY**  
**CRF-FU – CASE REPORT FORM – FOLLOW-UP**

J_6	J_6_1	Bronchoscopy done	0 - No	1 - Yes	**	If No Go to J_7
	J_6_2	Date	d   d   m   m	y   y   y   y	*	
	J_6_3	Airway compression	0 - No	1 - Yes	*	
	J_6_3_1	Trachea	0 - No	1 - Yes	*	
	J_6_4	Compression%				
	J_6_4_1	L Main Bronchus	0 - No	1 - Yes	*	
	J_6_5	Compression%				
	J_6_5_1	R Main Bronchus	0 - No	1 - Yes	*	
	J_6_6	Compression%				
	J_6_6_1	Bronchus intermedius	0 - No	1 - Yes	*	
J_6_7	Compression%					
J_6_7_1	Other	0 - No	1 - Yes	*		
J_6_7_2	Specify	Specify				
J_6_8	Compression%					
J_6_8_1	Other	0 - No	1 - Yes	*		
J_6_8_2	Specify	Specify				
J_6_9	Compression%					
J_6_9	Gland herniating	0 - No	1 - Yes	*		
J_7	J_7_1	Thoracic Surgery done	0 - No	1 - Yes	**	
J_7_2	Date	d   d   m   m	y   y   y   y	*		
J_7_3	Enucleation of glands	0 - No	1 - Yes	*		
J_7_3_1	Lobectomy	0 - No	1 - Yes	*		
J_7_3_1	Other procedure	Specify				

**K PATHOLOGY**

K_1	K_1_1	Fine Needle Aspirate done	0 - No	1 - Yes	**	If No Go to K_2
	K_1_2	Date	d   d   m   m	y   y   y   y	*	
	K_1_2	Where	1 Cervical LN			
	K_1_2		2 Axillary LN			
	K_1_2		-8 Other			
K_1_3	Specify	Specify				
K_2	K_1_3	Caseating granulomas	0 - No	1 - Yes	*	
	K_1_4	ZN	0 - Neg	1 - Pos	*	
	K_1_5	Other findings	Specify			
	K_2_1	Bone Marrow Biopsy done	0 - No	1 - Yes	**	
	K_2_2	Date	d   d   m   m	y   y   y   y	*	
K_2_3	Caseating granulomas	0 - No	1 - Yes	*		
K_2_4	ZN	0 - Neg	1 - Pos	*		
K_2_5	Culture	0 - Neg	1 - Pos	*		
K_2_5	Other findings	Specify				

**L Disease status**

1. TB-status	1	Remains case
	2	Remains control
	3	Changes from control to case
	4	Changes from case to control
2. Running diagnosis	1	
	2	
	3	

*Refers to overall diagnosis of the baseline episode and any ongoing health issues*

	RA CODE	DATE								SIGNATURE
COMPLETED BY		D	D	M	M	Y	Y	Y	Y	
QC BY PEER		D	D	M	M	Y	Y	Y	Y	
DATA CAPTURER 1		D	D	M	M	Y	Y	Y	Y	
DATA CAPTURER 2		D	D	M	M	Y	Y	Y	Y	

**TR: POOLING STUDY**  
**SDOC25 – TB CONTACT**

VISIT DATE	D	D	M	M	Y	Y	Y	Y
------------	---	---	---	---	---	---	---	---

STUDY VISIT	Baseline	Scheduled Visit	Unscheduled Visit			
CONTACT NUMBER*	1	2	3	4	5	6

\*Number for specific visit



In event of child having had contact with TB index case(s) in previous 12 months  
Fill in a separate form for each contact

\*\* Compulsory question  
\* Compulsory question depending on parent question  
Make use of jump instructions  
Make use of a black pen only

**A**

A_1	Could the index case be traced? A_1_1 If not, reason (>1 can apply)	1-Yes	0-No	**
1	No identifying details supplied			*
2	No record of index case at health care facilities			
3	No home address/ not living at address supplied			
-8	Other, specify:			

A_2	Is the index case confirmed or suspected	Confirmed	Suspect	**				
A_2_1	If confirmed, date of first positive bacteriology result if available	M	M	Y	Y	Y	Y	*

A_3	Is the index case on TB treatment?	1-Yes	0-No	-5-Unk	**			
A_3_1	If yes, when did TB treatment start (if available)	M	M	Y	Y	Y	Y	*
A_3_2	If yes, at which clinic/hospital (most recent) was/is the TB index case treated?							*

	If treated, is the index case adherent?	1-Yes	0-No	-5-Unk	**
--	---	-------	------	--------	----

A_4	Is the index case a re-treatment case?	1-Yes	0-No	-5-Unk	**
-----	--	-------	------	--------	----

A_4_1	If yes, how many prior TB episodes?	1	2	>2	-5	**
-------	-------------------------------------	---	---	----	----	----

A_5	Is the index case currently coughing?	1-Yes	0-No	-5-Unk	**
-----	---------------------------------------	-------	------	--------	----

A_6	What is the relation of the TB index case to the child?	1 Mother	2 Father	**
		3 Grandmother	4 Grandfather	
		5 Aunt	6 Uncle	
		7 Cousin	8 Sibling	
		9 Neighbour	10 Lodger	
		11 Caregiver other than family	-8 Other	

**TR: POOLING STUDY**  
**SDOC25 – TB CONTACT**

A_7	Is the index case the child's primary care giver?	1-Yes	0-No	-5-Unk	**			
A_8	Is there daily contact between the child and index case?	1-Yes	0-No	-5-Unk	**			
A_8_1	Where does the TB index case live?	1	In the house		*			
		2	Neighbouring house					
		-8	Other					
A_8_2	Does the index case sleep in the same room as child?	0 - No	1 – Yes	-5 Unknown	*			
A_8_3	Does the index case sleep in the same bed as the child?	0 - No	1 – Yes	-5 Unknown	*			
A_8_4	How many hours on average does the index case spend with the child per day?	1	0 – 4 Hrs		*			
		2	5 – 8 Hrs					
		3	9 – 12 Hrs					
		4	> 12 Hrs					
A_9	Did the child start IPT for this contact episode?	0 - No	1 – Yes	-5 Unknown	**			
A_9_1	If yes, date	M	M	Y	Y	Y	Y	*
A_9_2	At which clinic							

A\_10 TB bacteriological results of index

Date								Source	Smear	Culture	Xpert	Culture DST	Xpert DST
D	D	M	M	Y	Y	Y	Y						
D	D	M	M	Y	Y	Y	Y						
D	D	M	M	Y	Y	Y	Y						
D	D	M	M	Y	Y	Y	Y						
D	D	M	M	Y	Y	Y	Y						
D	D	M	M	Y	Y	Y	Y						
D	D	M	M	Y	Y	Y	Y						

**TR: POOLING STUDY**  
**SDOC25 – TB CONTACT**

D	D	M	M	Y	Y	Y	Y							
D	D	M	M	Y	Y	Y	Y							
D	D	M	M	Y	Y	Y	Y							
D	D	M	M	Y	Y	Y	Y							
D	D	M	M	Y	Y	Y	Y							

**A\_11 Other results**

Date								Source	Results
D	D	M	M	Y	Y	Y	Y		
D	D	M	M	Y	Y	Y	Y		
D	D	M	M	Y	Y	Y	Y		
D	D	M	M	Y	Y	Y	Y		
D	D	M	M	Y	Y	Y	Y		
D	D	M	M	Y	Y	Y	Y		
D	D	M	M	Y	Y	Y	Y		
D	D	M	M	Y	Y	Y	Y		
D	D	M	M	Y	Y	Y	Y		

**A\_12 Comments**

**TR: POOLING STUDY**  
**SDOC25 – TB CONTACT**

Source Options

1	Sputum
2	Induced Sputum
3	Gastric Aspirate
4	Pleural fluid
5	CSF
6	FNA
7	Lymph node
-8	Other
Specify	

Smear options

0	Negative
1	Scanty
10	1+
20	2+
30	3+
40	Insufficient
70	Leak
80	Empty
90	Contaminated
100	UTP

Xpert Options

0	Negative
1	Positive
2	Error
3	Invalid
-5	Unknown

Culture Options

1	MTB
2	NTM
90	Contaminated
-5	Unknown

Culture DST

1	DS TB
2	Rif Mono - R
3	INH Mono - R
4	MDR TB
5	MDR+ Of - R
6	MDR+Ami-R
7	XDR
-5	Unknown

Xpert DST

1	Rif sensitive
2	Rif resistant
3	Rif indeterminate

SDOC9												
<b>STANDARDISED CXR REPORT FORM</b>												
VISIT	BL	M2	M6	UNSC								
				<b>CHILD BARCODE</b>								
<b>A</b>												
1	Reader	<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 33%;">1 - RPG</td> <td style="width: 33%;">2 - HSS</td> <td style="width: 33%;">3 - OTHER</td> </tr> <tr> <td colspan="3">SPECIFY OTHER</td> </tr> </table>			1 - RPG	2 - HSS	3 - OTHER	SPECIFY OTHER				
1 - RPG	2 - HSS	3 - OTHER										
SPECIFY OTHER												
2	CXR Date	<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 12.5%;">D</td> <td style="width: 12.5%;">D</td> <td style="width: 12.5%;">M</td> <td style="width: 12.5%;">M</td> <td style="width: 12.5%;">Y</td> <td style="width: 12.5%;">Y</td> <td style="width: 12.5%;">Y</td> <td style="width: 12.5%;">Y</td> </tr> </table>			D	D	M	M	Y	Y	Y	Y
D	D	M	M	Y	Y	Y	Y					
3	Quality of CXR	<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td colspan="2" style="text-align: center;">0 - No</td> <td colspan="2" style="text-align: center;">1 - Yes</td> </tr> <tr> <td style="width: 25%;">0 - No</td> <td style="width: 25%;">1 - Yes</td> <td style="width: 25%;">2 - Not Done</td> <td style="width: 25%;"></td> </tr> </table>			0 - No		1 - Yes		0 - No	1 - Yes	2 - Not Done	
0 - No		1 - Yes										
0 - No	1 - Yes	2 - Not Done										
4	Normal CXR	<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 33%;">0 - No</td> <td style="width: 33%;">1 - Yes</td> <td style="width: 33%;">3 - Not Acceptable</td> </tr> </table>			0 - No	1 - Yes	3 - Not Acceptable					
0 - No	1 - Yes	3 - Not Acceptable										
<b>B</b>												
PARENCHYMA		(tick applicable boxes)										
Alveolar (Consolidation)	Y	N			RUL	RML	RLL	LUL	LING	LLL	Segm	Lobar
Ghon focus	Y	N			RUL	RML	RLL	LUL	LING	LLL	Segm	Lobar
Expansile	Y	N			RUL	RML	RLL	LUL	LING	LLL	Segm	Lobar
Bronchopneumonic	Y	N			RUL	RML	RLL	LUL	LING	LLL	Segm	Lobar
Cavity	Y	N			RUL	RML	RLL	LUL	LING	LLL		
Interstitial Pattern	Y	N			Peri-hilar		Peripheral		Global		Right	Left
Miliary infiltrate	Y	N			Peri-hilar		Peripheral				Right	Left
Collapse	Y	N			RUL	RML	RLL	LUL	LING	LLL	Segm	Lobar
Hyperinflation (Lobar/segmental)	Y	N			RUL	RML	RLL	LUL	LING	LLL	Segm	Lobar
Hyperinflation (Generalised )	Y	N										
Calcification (lung)	Y	N			RUL	RML	RLL	LUL	LING	LLL	Segm	Lobar
Fibrosis	Y	N			RUL	RML	RLL	LUL	LING	LLL	Segm	Lobar
Peri-hilar streakiness	Y	N			Right		Left					
NODES												
Peri-hilar	Y	N	U		Right		Left		Subcarinal		Unsure	
Paratracheal	Y	N	U		Right		Left		Unsure			
Calcification (nodes)	Y	N			Peri-hilar		Paratracheal					
AIRWAYS												
Tracheal compression	Y	N			Right		Left					
Bronchial compression	Y	N			Right		Left					
PLEURA												
Effusion	Y	N			R	L	Small		Large		Loculated	
Thickening	Y	N			R	L						
Pneumothorax	Y	N			R	L						
CARDIAC												
Cardiac Enlargement	Y	N	U						Pericardial Effusion		Y N U	

6	Certainty of Active diagnosis	1 Certain TB	2 Uncertain TB	3 Not TB				
<b>C - Certain TB</b>								
1. If <b>Certain Active TB</b> , indicate TB disease classification (check all that apply)								
	1	Uncomplicated LN Disease						
	2	Complicated LN Disease						
	3	Expansile pneumonia						
	4	Ghon focus						
	5	Miliary TB						
	6	Pleural effusion						
	7	Cavities						
	8	Adult Type TB						
	9	Calcification						
	-8	Other _____						
<b>D - Not TB</b>								
1. If <b>Not Active TB</b> , Indicate Alternative diagnosis (circle all that apply)								
	1	Lobar pneumonia						
	2	Interstitial pneumonia						
	3	Bronchopneumonia						
	4	LAWO						
	5	Congenital malformation						
	6	LIP						
	7	Cavity not TB						
	8	Bronchiectasis						
	9	Calcification (Previous TB)						
	-8	Other, specify						
<b>E Quantitative Assessment if CXR abnormal</b>								
1_1	Number of zones with parenchymal pathology	0	1	2	3	4	5	6
1_2	Indicate zone							
1_3	Extent of pathology	1 = RUL		2 < RUL		3 > RUL		
2	Any <b>nodal airway compression</b> airway	0 - No			1 - Yes			
TREATMENT RESPONSE STUDY			SDOC9 - PAGE 2 of 3			VER 1.0 22 July 2012		

- 2.1 If Yes, bronchial compression
- 2.2 If Yes, tracheal compression

1 - Right	2 - Left	3 - Both
0 - No		1 - Yes

- 1 Compared to previous CXR, current CXR

0	Unchanged
1	Improved
2	Deteriorated
3	Different Pathology
4	Uncertain

- 2 Notes and comments for follow-up

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	RA CODE	DATE								SIGNATURE
STUDY DOCTOR		D	D	M	M	Y	Y	Y	Y	
QC BY PEER (ON SITE)		D	D	M	M	Y	Y	Y	Y	
DATA TYPIST 1		D	D	M	M	Y	Y	Y	Y	
DATA TYPIST 2		D	D	M	M	Y	Y	Y	Y	



**TREATMENT RESPONSE  
SDOC11 –PROTOCOL DEVIATION**

REPORTED DATE	D	D	M	M	Y	Y	Y	Y
---------------	---	---	---	---	---	---	---	---

VISIT	Baseline	Scheduled Visit	Unscheduled Visit
-------	----------	-----------------	-------------------



PLEASE COMPLETE A NEW FORM FOR EACH PROTOCOL DEVIATION, FOR EACH SUBJECT

Protocol Deviation Date 

D	D	M	M	Y	Y	Y	Y
---	---	---	---	---	---	---	---

Did the deviation result in an adverse event? 

0 – No	1 - Yes
--------	---------

Did the deviation result in a serious adverse event? 

0 – No	1 - Yes
--------	---------

Did the deviation result in subject termination of study follow - up? 

0 – No	1 - Yes
--------	---------

Brief description of deviation(if space not sufficient continue overleaf sign and date every page)

*If contraindication to a procedure, explain in detail what contraindication existed.*

Reason for deviation

1	Subject refusal
2	Unable to trace subject
3	Contra-indication to procedure
3.1	Specify:
4	Laboratory error
5	Procedural error
6	Staff error

TREATMENT RESPONSE SDOC11 –PROTOCOL DEVIATION																																																																
Category of deviation	<table border="1" style="width: 100%; border-collapse: collapse;"> <tr><td style="width: 20px; text-align: center;">7</td><td>Investigator decision</td></tr> <tr><td style="text-align: center;">-8</td><td>Specify</td></tr> <tr><td style="text-align: center;">1</td><td>Eligibility / Enrolment</td></tr> <tr><td style="text-align: center;">2</td><td>Consent</td></tr> <tr><td style="text-align: center;">3</td><td>Sampling</td></tr> <tr><td style="text-align: center;">4</td><td>Missed Visit</td></tr> <tr><td style="text-align: center;">-8</td><td>Specify</td></tr> </table>									7	Investigator decision	-8	Specify	1	Eligibility / Enrolment	2	Consent	3	Sampling	4	Missed Visit	-8	Specify																																									
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Record steps to resolve and avoid recurrence of deviation																																																																
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DATA CAPTURER 2		D	D	M	M	Y	Y	Y	Y																																																							
PAGE 2 OF 2					Treatment Response Study																																																											

**TREATMENT RESPONSE**  
**SDOC17 – ADHERENCE ASSESSMENT**

DATE	D	D	M	M	Y	Y	Y	Y
VISIT	Baseline		Scheduled Visit		Unscheduled Visit			



\*\* Compulsory question  
 \* Compulsory question depending on parent question  
 Make use of jump instructions  
 Make use of a black pen only

**Adherence Questionnaire for research study: "Diagnostic yield and treatment response in childhood intrathoracic tuberculosis: effect of disease severity". To be completed only for children on TB treatment or prophylaxis**

**A IDENTIFYING NON-ADHERENT BEHAVIOUR**

A\_1 Do you ever forget to give your child the TB medicine? 1 – Yes   0 – No   Uncertain

A\_2 If the child feels well, do you sometimes **not** give your child the TB medicine? 1 – Yes   0 – No   Uncertain

A\_3 If the child does not feel well / is not well, do you sometimes **not** give your child the TB medicine? 1 – Yes   0 – No   Uncertain

**B QUANTIFYING ADHERENCE BY RECALL**

B\_1 In the past 3 days, how many doses of TB medicines have you **not** given your child? [ ] \*\*  
 B\_1\_1 Percentage doses given [ ]

B\_2 In the past month (or 2 weeks if visit 1 or 2), how many days have you **not** given your child the TB medicine? [ ] \*\*  
 B\_2\_1 Percentage days in which treatment was given [ ]

B\_3 Have you ever missed one of your child's TB doses? 1 – Yes   0 – No   Uncertain  
 B\_3\_1 If yes: When was the last time you missed one of your child's TB doses? D D M M Y Y Y Y  
 B\_3\_2 Percentage doses given [ ]

B\_4 Cumulative adherence (average of 3 percentages above) [ ]

**C Dosage calculation**

Put an X at the point showing your best guess about how much medication you have given your child in the past month. An X at 100 means you've given all the doses, at 0 means you've given none of the doses

C\_1

100		
90		
80		
70		
60		
50		
40		
30		
20		
10		
0		

**TREATMENT RESPONSE**  
**SDOC17 – ADHERENCE ASSESSMENT**

If cumulative adherence is <90% or any single adherence measure is <80%, proceed to the below questionnaire:

1. (If applicable) Which drug(s) is/are the most difficult to give?  
(Here you should have a drop down list of all possible TB drugs)
2. Why do you at times not give the medication? (For each have a Y/N option)
  - a) I just forget
  - b) I run out of medicine and don't go back to clinic for more
  - c) The child doesn't like the medicine
  - d) The child vomits after the medicine
  - e) The medicine has bad side effects. Please specify.
  - f) I am too busy.
  - g) I don't believe the medicines help.
  - h) The child is well.
  - i) There was a change in my routine. Please specify (e.g. travel, job changed, someone else looks after child, hospitalization)
  - j) Other, specify

**Note for the researcher**

Engage the participants in ways of improving adherence on each of the identified issues. Examples below.

- a) Reminders: cell phone, alarm clock, radio/TV. Supporter at home. Link with daily activity.
- b) Counsel. Engage treatment supporter.
- c) Positive rewards. Precede or follow medicine with sweet food.
- d) Split dose until child is used to medicine. Follow with food. Give before bedtime.
- e) Thorough examination. Perceived vs. real side effects. See c) and d).
- f) Find suitable time for giving medication. Identify alternative carer who can administer medicines.
- g) Engage and counsel.
- h) Counsel re danger of drug resistance and rapid progression of untreated TB in children.
- i) Back-up arrangements.

**Comments on counselling undertaken by researcher and follow-up plans: (Free text box)**

**TREATMENT RESPONSE**  
**SDOC17 – ADHERENCE ASSESSMENT**

**Notes to researcher:**

Calculating percentage adherence:

2.1 Doses given /doses that should have been givenx100

2.2 Days given /days in the evaluation periodx100

2.3 Doses given from day doses was last missed / expected doses x 100 e.g. if visit day is Friday and last dose missed was Monday, doses given = 4, doses expected = 5, so 4/5 x 100 = 80%

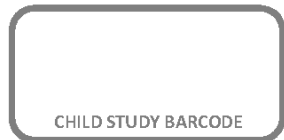
Final adherence measured = 2.1 + 2.2 + 2.3(%) / 3

	RA CODE	DATE								SIGNATURE
COMPLETED BY		D	D	M	M	Y	Y	Y	Y	
QC BY PEER		D	D	M	M	Y	Y	Y	Y	
DATA CAPTURER 1		D	D	M	M	Y	Y	Y	Y	
DATA CAPTURER 2		D	D	M	M	Y	Y	Y	Y	



DTTC Treatment Response Study

# LAB1 – Laboratory CRF



ONLY FOR RESPIRATORY and STOOL (not for blood, not for urine)

1 form per specimen or pooled specimen. The form must be printed on 1 page (both sides)

<b>THIS SECTION TO BE COMPLETED BY CLINICAL STAFF – Bring to DTTC Mini Lab (Rm0073)</b>			
SAMPLE BARCODE			
SAMPLE BARCODE #2 if pooled/stool			
SAMPLE BARCODE #3 if pooled/stool			
<b>ALL OTHER SECTIONS for LABORATORY USE ONLY:</b>			
NHLS SOURCE <input type="checkbox"/> A_Gastric Aspirate <input type="checkbox"/> G_BAL <input type="checkbox"/> B_NPA <input type="checkbox"/> H_FNA <input type="checkbox"/> C_Sputum <input type="checkbox"/> I_Pleural fluid <input type="checkbox"/> D_Induced Sputum <input type="checkbox"/> J_Other: <input type="checkbox"/> E_“Pooled specimen” <input type="checkbox"/> F_Stool <input type="checkbox"/> K_GA OMNIgene		NHLS ST # or Xpert NHLS Lab Number (for Stool)	
		DATE received NHLS (DD/MM/YYYY)	___/___/___
		Time received NHLS (HH: MM)	___:___
No results <input type="checkbox"/> [See Requisition Form for more detail]			
<b>AURAMINE [Smear]</b>			
<input type="checkbox"/> A_Neg <input type="checkbox"/> D_Pos:2+ <input type="checkbox"/> B_Scanty <input type="checkbox"/> E_Pos:3+ <input type="checkbox"/> C_Pos:1+ <input type="checkbox"/> Z_Not Done			
<b>XPRT MTB/RIF:</b>			
GENEXPERT [GeneXpert MTB/RIF <u>FINAL</u> result]	<input type="checkbox"/> A_Neg <input type="checkbox"/> B_Pos <input type="checkbox"/> C_Invalid <input type="checkbox"/> D_Error <input type="checkbox"/> E_No Result <input type="checkbox"/> Z_Not Done	FREQUENCY [GeneXpert MTB/RIF semi-quantitative result]	<input type="checkbox"/> A_V.Low <input type="checkbox"/> B_Low <input type="checkbox"/> C_Medium <input type="checkbox"/> D_High <input type="checkbox"/> E_No Result <input type="checkbox"/> Z_Not Done
INITIAL GENEXPERT [If GENEXPERT was repeated, what was initial Xpert result]	<input type="checkbox"/> A_Neg <input type="checkbox"/> B_Pos <input type="checkbox"/> C_Invalid <input type="checkbox"/> D_Error <input type="checkbox"/> E_No Result <input type="checkbox"/> Z_Not Done	GeneXpert RIF [GeneXPRT RIF res ult]	<input type="checkbox"/> A_Susc <input type="checkbox"/> B_Res <input type="checkbox"/> C_Indeterminate <input type="checkbox"/> Z_Not Done

QC done by lab team:	RA CODE	DATE								SIGNATURE
PAGE 1 COMPLETED BY		D	D	M	M	Y	Y	Y	Y	
PAGE 1 QC BY PEER		D	D	M	M	Y	Y	Y	Y	
PAGE 1 DATA CAPTURE 1		D	D	M	M	Y	Y	Y	Y	
PAGE 1 DATA CAPTURE 2		D	D	M	M	Y	Y	Y	Y	

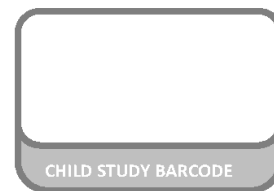
MGIT Culture			
MGIT [MGIT <u>FINAL</u> result]	<input type="checkbox"/> A_Pos_MTB <input type="checkbox"/> B_Neg <input type="checkbox"/> C_Contam <input type="checkbox"/> D_Pos_NTM <input type="checkbox"/> Z_Not Done	TTP [Report only if Pos for MTB, tick "Not Applicable" if not]	____ Days ____ Hours <input type="checkbox"/> Not Applicable
Details of MGIT Culture (not on NHLS report)			
ZN [MGIT ZN]	<input type="checkbox"/> A_Neg <input type="checkbox"/> B_Pos <input type="checkbox"/> Z_Not Done	Hain CM result <input type="checkbox"/> A_Pos for MTBC <input type="checkbox"/> B_Pos for NTM <input type="checkbox"/> C_Pos MTBC+ NTM	<input type="checkbox"/> D_NTM identified or other result: _____ <input type="checkbox"/> Z_Not Done
BioLine [MPT64Ag BIOLINE]	<input type="checkbox"/> A_Neg <input type="checkbox"/> B_Pos <input type="checkbox"/> Z_Not Done	BAP	<input type="checkbox"/> A_Growth <input type="checkbox"/> B_No Growth <input type="checkbox"/> Z_Not Done
Drug Susceptibility results: Hain Test			
LPA MTB [Hain MTBDRplus identification]	<input type="checkbox"/> A_Neg <input type="checkbox"/> B_Pos <input type="checkbox"/> C_Refer to other LPA <input type="checkbox"/> Z_Not Done	LPA INH [Hain MTBDRplus INH result]	<input type="checkbox"/> A_Susc <input type="checkbox"/> B_Res <input type="checkbox"/> C_Indeterminate <input type="checkbox"/> Z_Not Done
LPA RIF [Hain MTBDRplus RIF result]	<input type="checkbox"/> A_Susc <input type="checkbox"/> B_Res <input type="checkbox"/> C_Indeterminate <input type="checkbox"/> Z_Not Done	LPA INH mutation [Hain MTBDRplus INH mutation] (If INH R, is R to INH due to a inhA or katG mutation?)	<input type="checkbox"/> A_inhA mutation detected <input type="checkbox"/> B_katG mutation detected <input type="checkbox"/> C_both inhA and katG mutations detected <input type="checkbox"/> Z_Not Done
Drug Susceptibility results: 2 <sup>nd</sup> Line Testing			
AMIKACIN (2 <sup>nd</sup> Line Testing done at NHLS GP)	<input type="checkbox"/> A_Susc <input type="checkbox"/> B_Res <input type="checkbox"/> C_Indeterminate <input type="checkbox"/> Z_Not Done	OFLOXACIN (2 <sup>nd</sup> Line Testing done at NHLS GP)	<input type="checkbox"/> A_Susc <input type="checkbox"/> B_Res <input type="checkbox"/> C_Indeterminate <input type="checkbox"/> Z_Not Done
OTHER DST performed	_____		
<b>Other</b>			
COMMENTS (e.g. other LPA ST)	_____		

QC done by lab team:	RA CODE	DATE								SIGNATURE
PAGE 2 COMPLETED BY		D	D	M	M	Y	Y	Y	Y	
PAGE 2 QC BY PEER		D	D	M	M	Y	Y	Y	Y	
PAGE 2 DATA CAPTURE 1		D	D	M	M	Y	Y	Y	Y	
PAGE 2 DATA CAPTURE 2		D	D	M	M	Y	Y	Y	Y	



DTTC Treatment Response Study

**REQ1 - DTTC Requisition Form**



Date taken	D	D	M	M	Y	Y	Y	Y
Time taken	H	H	H	M	M			
Taken by	RA CODE							

Visit	0	0_Baseline	Specimen Type	9	9_Serum for biomarker
	1	1_Scheduled		5	5_Urine
	-5	-5_Unscheduled		6	6_Stool
Patient	1	1_Inpatient	Collection Method / Tube	1	1_Serum in SST Tube
	2	2_Outpatient		2	2_Urine collected in container (without using bag)
Suppository used (for stool)	0	0_No	3	3_Urine collected using bag	
	1	1_Yes	4	4_Stool collected in container	
	-5	-5_Not applicable	5	5_Stool collected in waterproof with diaper	
			6	6_Stool collected in diaper (no waterproof)	
			7	7_Stool collected in urine bag with diaper	
		8	8_Stool collected in cling film over toilet		
		9	9_Stool collected in potty/bedpan		

Sample Barcode #1	Sample Barcode #2 (only for stool portion)	Sample Barcode #3 (only for stool portion)
<div style="border: 1px solid black; height: 30px;"></div>	<div style="border: 1px solid black; height: 30px;"></div>	<div style="border: 1px solid black; height: 30px;"></div>

**FOLLOWING SECTION FOR LAB USE ONLY:**

Sample Barcode #1 Use:	Sample Barcode #2 Use:	Sample Barcode #3 Use:
Reject: leaked	Reject: leaked	Reject: leaked
Reject: time from collection	Reject: time from collection	Reject: time from collection
Other (detail in comments)	Other (detail in comments)	Other (detail in comments)
Comment:	Comment:	Comment:

**FOLLOWING SECTION FOR LAB USE ONLY: STOOL SAMPLES ONLY**

Sample Barcode #1 Use:	Sample Barcode #2 Use:	Sample Barcode #3 Use:
Stool Processed	Stool Processed	Stool Processed
Stool Stored	Stool Stored	Stool Stored

Stool weight processed	<input type="text"/>	g	Stool Appearance	Solid	Sticky
Processed by: (RA CODE)	<input type="text"/>			Semi solid	Liquid
Processed date:	<input type="text"/>		GXP Number	<div style="border: 1px solid black; height: 30px;"></div>	
Comment (stools):	<input type="text"/>				

	RA CODE	DATE								SIGNATURE
COMPLETED BY		D	D	M	M	Y	Y	Y	Y	
QC BY LAB TECH.		D	D	M	M	Y	Y	Y	Y	
DATA CAPTURE 1		D	D	M	M	Y	Y	Y	Y	
DATA CAPTURE 2		D	D	M	M	Y	Y	Y	Y	



TYGERBERG HOSPITAL DEPARTMENT OF PATHOLOGY, DIVISION OF MEDICAL MICROBIOLOGY																																													
LAB TRIAL NO.					Paediatric TB Treatment Response Trial																																								
Location	S	T	5	0	4	2	Folder No.			_____																																			
Study: Treatment Response							Surname			_____																																			
Investigator: Dr. E. Walters (021) 938 9177							First name			_____																																			
Co-ordinator: J. Workman (021) 938 9834							DOB			D	D	M	M	Y	Y	Sex:	_____																												
Account: 12ZTU000197							[For DTTC: Put TYG Hospital Number, except Day1 Resp Samples Situation 1 & 2: Child Barcode]																																						
DTTC Sample barcode			DTTC Sample barcode #2 (only if pooled)			DTTC Sample barcode #3 (if pooled)																																							
_____			_____			_____																																							
Date taken:*			D	D	M	M	Y	Y	[NHLS Code]			[DTTC Specimen Type Code for DTTC use only]																																	
Time taken:*			H	H	M	M				↓			↓																																
Taken by:*			RA CODE			[RGA2]			Gastric Aspirate (GA)			POOL (For DTTC Use Only):																																	
Request by: Dr. E. Walters MP0545198							[RNA]			NPA			<input type="checkbox"/> Gastric aspirate <input type="checkbox"/> NPA <input type="checkbox"/> Exp. Sputum early AM <input type="checkbox"/> Exp. Sputum Spot <input type="checkbox"/> Ind. Sputum (with suction) <input type="checkbox"/> Ind. Sputum (no suction) <input type="checkbox"/> Pooled specimen: indicate ALL specimens in pool																																
Visit:			<input type="checkbox"/> Baseline <input type="checkbox"/> Scheduled <input type="checkbox"/> Unscheduled			[RSP]			Expectorated sputum early AM			<input type="checkbox"/> Exp. Sputum early AM <input type="checkbox"/> Exp. Sputum Spot <input type="checkbox"/> Ind. Sputum (with suction) <input type="checkbox"/> Ind. Sputum (no suction)																																	
Patient:			<input type="checkbox"/> Inpatient <input type="checkbox"/> Outpatient [RSP with "pooled" in comments]			[RSP1]			Induced sputum (with suction)																																				
Last meal before sampling:*			Date			D	D	M	M	Y	Y	For GA Specimens Only:																																	
Time			H	H	M	M				pH of raw specimen			_____																																
Specimen volume*			<input type="checkbox"/> <1 ml <input type="checkbox"/> 1 - 2 ml <input type="checkbox"/> >2 ml			Macroscopic appearance:*			(Indicate X for any that apply)			Was bicarb added? Y / N																																	
If >2ml, specify volume: _____			<input type="checkbox"/> Frank Blood <input type="checkbox"/> Blood streaks <input type="checkbox"/> Blood specks <input type="checkbox"/> Watery <input type="checkbox"/> Mucoid <input type="checkbox"/> Cloudy/milky <input type="checkbox"/> Purulent									If yes, volume added (ml) _____																																	
												pH of specimen after bicarb _____																																	
REFERRAL TO TYG MICROBIOLOGY Lab (Come: 021 938-4031)										Tb Microscopy, Culture & GeneXpert			Patient Profile: TBCUL PCRGXP																																
<b>For NHLS Use Only:</b>					<table border="0" style="width:100%;"> <tr> <td style="width: 33%;">Direct Microscopy</td> <td style="width: 33%;">TB Suspect</td> <td style="width: 33%;">No</td> <td style="width: 33%;">Hain MTBDRplus on Positive Culture</td> <td style="width: 33%;">TB Suspect</td> <td style="width: 33%;">Yes</td> </tr> <tr> <td>Concentrated Auramine Smear</td> <td>Yes</td> <td>No</td> <td>Susceptibility Testing (MGIT)</td> <td>No</td> <td>No</td> </tr> <tr> <td>Direct Hain MTBDRplus</td> <td>No</td> <td>No</td> <td>2nd Line DST for MDR Isolates</td> <td>Yes</td> <td>Yes</td> </tr> <tr> <td>GeneXpert on sample (decontaminated)</td> <td>Yes</td> <td>No</td> <td>Routine Identification MOTTs</td> <td>On request</td> <td>On request</td> </tr> <tr> <td>MGIT Culture</td> <td>Yes</td> <td>No</td> <td>Storage of isolate (-70)</td> <td>No</td> <td>No</td> </tr> <tr> <td>Storage of Specimen</td> <td>No</td> <td>No</td> <td colspan="3">↳ *Keep for DTTC Lab Team: Come Bosch*</td> </tr> </table>					Direct Microscopy	TB Suspect	No	Hain MTBDRplus on Positive Culture	TB Suspect	Yes	Concentrated Auramine Smear	Yes	No	Susceptibility Testing (MGIT)	No	No	Direct Hain MTBDRplus	No	No	2nd Line DST for MDR Isolates	Yes	Yes	GeneXpert on sample (decontaminated)	Yes	No	Routine Identification MOTTs	On request	On request	MGIT Culture	Yes	No	Storage of isolate (-70)	No	No	Storage of Specimen	No	No	↳ *Keep for DTTC Lab Team: Come Bosch*		
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Treatment Response Study - NHLS Form - REQ2 - Version 2.0 - 28 January 2018										Page 1 of 1																																			



DTTC Treatment Response Study

# SAM 2 Sample Transport Log

1 FORM per TRANSPORT BOX. 1 BOX for samples with same transport temperature and same destination.

Sample	Temp.	Destination
Resp.	2-8°C	NHLS TB Lab 9 <sup>th</sup> Floor
NPA	2-8°C	Virology Lab 8 <sup>th</sup> Floor Clinical Building
Stool	2-8°C	DTTC Mini Lab K0073
Urine	2-8°C	DTTC Mini Lab K0073
Blood	18-25°C	DTTC Mini Lab K0073
OMNI gene	18-25°C	NHLS TB Lab 9 <sup>th</sup> Floor

No	CHILD STUDY BARCODE	DTTC SAMPLE BARCODE (1 barcode per line)
1		SAMPLE BACRODE
2		SAMPLE BACRODE
3		SAMPLE BACRODE
4		SAMPLE BACRODE
5		SAMPLE BACRODE
6		SAMPLE BACRODE

DEPARTURE SITE (indicate with X):							ARRIVAL SITE (indicate with X):						
1_Tygerberg Hospital G Ground							1_NHLS Tygerberg TB Lab (9 <sup>th</sup> Floor)						
2_Participant Home							2_NHLS Virology (8 <sup>th</sup> Floor- Clinical Building)						
3_Other, specify:							3_DTTC Mini Lab Room K0073						
							5_Other, specify:						
Received by: _____													
Date departing	D	D	M	M	Y	Y	Date arriving	D	D	M	M	Y	Y
Time departing	H	H	H	M	M		Time arriving	H	H	H	M	M	
Temperature departing						°C	Temperature arriving						°C

	RA CODE	DATE								SIGNATURE
TRANSPORTED BY		D	D	M	M	Y	Y	Y	Y	
QC BY LAB TECH.		D	D	M	M	Y	Y	Y	Y	
DATA CAPTURE 1		D	D	M	M	Y	Y	Y	Y	
DATA CAPTURE 2		D	D	M	M	Y	Y	Y	Y	

Treatment Response Study - Sample Transport Log – Version 1.2  
17 August 2016



# Appendix E

## Ethics Approval

### E.1 Original Approval



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05 October 2011

**MAILED**

Dr E Walters  
Department of Paediatrics  
Tygerberg Hospital  
Francie van Zijl Drive  
Parow Valley  
7500

Dear Dr Walters

**Diagnostic Yield and Treatment Response in Childhood Intra-Thoracic Tuberculosis: Effect of Disease Severity.**

**ETHICS REFERENCE NO: N1109/282**

**RE : APPROVAL**

It is a pleasure to inform you that the Health Research Ethics Committee has approved the above-mentioned project at a meeting on 5 October 2011, including the ethical aspects involved, for a period of one year from this date.

This project is therefore now registered and you can proceed with the work. Please quote the above-mentioned project number in ALL future correspondence.

Please note a template of the progress report is obtainable on [www.sun.ac.za/rds/](http://www.sun.ac.za/rds/) and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly and subjected to an external audit.

Translations of the consent document in the languages applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372  
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health ([healthres@pgwc.gov.za](mailto:healthres@pgwc.gov.za) Tel: +27 21 483 9907) and Dr Hélène Visser at City Health ([Helene.Visser@capetown.gov.za](mailto:Helene.Visser@capetown.gov.za) Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

Approval Date: 5 October 2011

Expiry Date: 5 October 2012

18 November 2011 11:38

Page 1 of 2



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Yours faithfully

**MS CARLI SAGER**

**RESEARCH DEVELOPMENT AND SUPPORT**

Tel: +27 21 938 9140 / E-mail: [carlis@sun.ac.za](mailto:carlis@sun.ac.za)

Fax: +27 21 931 3352

18 November 2011 11:38

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## E.2 Latest Approval



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### Ethics Letter

**21-Feb-2018**

**Ethics Reference #: N11/09/282**

**Title:** Diagnostic yield and treatment response in childhood intrathoracic tuberculosis: effect of disease severity and HIV infection

Dear Dr Elisabetta Walters,

At a review meeting of HREC2 on 21 February 2018, the following progress report was reviewed and approved:

**Progress Report dated 19 January 2018.**

The approval of the research project is extended for a further year.

**Approval Date: 21 February 2018**

**Expiry Date: 20 February 2019**

Kindly be reminded to submit progress reports two (2) months before expiry date.

#### Where to submit any documentation

Kindly submit **ONE HARD COPY** to Elvira Rohland, RDSD, Room 5007, Teaching Building, and **ONE ELECTRONIC COPY** to [ethics@sun.ac.za](mailto:ethics@sun.ac.za).

Please remember to use your **Ethics Reference Number (N11/09/282)** on any documents or correspondence with the HREC concerning your research protocol.

National Health Research Ethics Council (NHREC) Registration Numbers: REC-130408-012 for HREC1 and REC-230208-010 for HREC2

Federal Wide Assurance Number: 00001372

Institutional Review Board (IRB) Number: IRB0005240 for HREC1

Institutional Review Board (IRB) Number: IRB0005239 for HREC2

The Health Research Ethics Committee complies with the SA National Health Act No. 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of



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Faculty of Medicine and Health Sciences



Afdeling Navorsingsontwikkeling, Opleiding, Bestuur, Toelating en Support Division

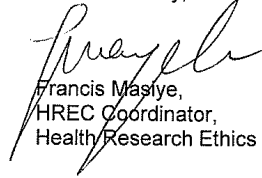
Posbus/PO Box 241 • Cape Town 8000 • Suid-Afrika/South Africa  
Tel: +27 (0) 21 938 9677



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Helsinki and the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles, Structures and Processes 2015 (Departement of Health).

Yours sincerely,



Francis Mashe,  
HREC Coordinator,  
Health Research Ethics Committee

STELLENBOSCH UNIVERSITY  
Health Research Ethics Committee  
  
21 FEB 2018  
  
STELLENBOSCH UNIVERSITEIT  
Gesondheidsnavorsing Etiekcommittee



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Faculty of Medicine and Health Sciences



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## E.3 PhD Approval



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### Acknowledgement Letter Documentation

13 March 2018

**HREC Reference #: N11/09/282**

**Title: Diagnostic Yield and Treatment Response in Childhood Intra-Thoracic Tuberculosis: Effect of Disease Severity**

Dear Dr Elisabetta Walters,

This letter confirms that the above referenced research project supports your PhD study entitled “**Innovative strategies to improve the diagnosis of intrathoracic tuberculosis in children**”. The parent research project received annual ethics renewal at a review meeting of HREC2 on 21 February 2018 and the approval of the research project is extended for another year as follows:

**Approval Date: 21 February 2018**

**Expiry Date: 20 February 2019**

Please proceed with analyses for your doctoral study and kindly be reminded to submit progress reports two (2) months before the expiry date.

#### Where to submit any documentation

Kindly submit **ONE HARD COPY** to Elvira Rohland, RDSD, Room 5007, Teaching Building, and **ONE ELECTRONIC COPY** to [ethics@sun.ac.za](mailto:ethics@sun.ac.za).

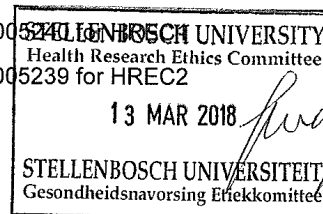
Please remember to use your **HREC Reference Number (N11/09/282)** on any documents or correspondence with the HREC concerning your research protocol.

National Health Research Ethics Council (NHREC) Registration Numbers: REC-130408-012 for HREC1 and REC-230208-010 for HREC2

Federal Wide Assurance Number: 00001372

Institutional Review Board (IRB) Number: IRB00052416 for HREC1

Institutional Review Board (IRB) Number: IRB0005239 for HREC2



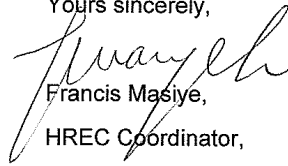




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The Health Research Ethics Committee complies with the SA National Health Act No. 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki and the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles, Structures and Processes 2015 (Department of Health).

Yours sincerely,



Francis Masiye,

HREC Coordinator,

Health Research Ethics Committee

STELLENBOSCH UNIVERSITY  
Health Research Ethics Committee

13 MAR 2018

STELLENBOSCH UNIVERSITEIT  
Gesondheidsnavorsing Eetiekcommittee

## Appendix F

### **Acknowledgements**

I am extremely grateful and privileged to be part of the Desmond Tutu TB Centre. The support I have received at all levels, the opportunities and the exciting blend of different people and ideas that I experience every day are truly unique.

I am especially grateful to my two extraordinary supervisors, Anneke Hesselning and Robert Gie. Anneke, your breadth of knowledge and your enthusiasm for pursuing new opportunities and collaborations are unparalleled. Thank you for providing consistent and valuable mentorship, and for always being willing to help and support.

Rob, I have valued your support and encouragement immensely. I always enjoy exchanging thoughts and ideas with you; you always provide a fresh and unique angle. Thank you for challenging me and believing in me.

Very special thanks to my two colleagues and friends, Marieke and Megan, who have taken on so much to allow me to complete this work. Marieke, thank you for believing this massive study was a good idea, and for helping in so many ways to make it happen. I have learnt so much from your ability to work efficiently and effectively, and to see things from different perspectives. Megan, thank you for always being such a positive force within the group and for challenging my clinical skills.

To all my work friends and colleagues, particularly Adrie, Karen, Angela and Helena: thank you for your support and friendship.

I thank all those involved in the many aspects of this study, without whom this work would not have happened. Thank you to Julie, Pierre and Simon, not only for the innumerable hours spent reading chest x-rays, but for doing so willingly, and for always being available to provide guidance, advice and practical help with challenging clinical cases.

I thank all the support staff who were involved with this study, including all the nurses, counsellors, drivers and data clerks. In particular, I want to thank Eloise Playandi, who started this study with me, when everything was new and daunting: thank you for your enthusiasm and kindness.

This study has a large laboratory component, and would not have been possible without a strong lab team. Thank you to Anne-Marie, for providing countless insights into the relevant microbiology issues pertaining to the study. Your knowledge and meticulous attention to detail are extraordinary; I have learnt a great deal from you. Thank you also for the PhD comic strips that kept the morale up when the going got tough. Thank you to Corné, for doing the bulk of the lab work for this study, and for continuing to be enthusiastic about stool-based diagnosis for paediatric TB! Thank you for always being so prompt to assist with many lab queries, and for many hours auditing the lab results database.

I have been assisted greatly by our data team, especially Rory and Taryn. Thank you for the many hours spent to ensure high quality data.

I wish to thank the hospital staff for their assistance, particularly G Ground staff, Sr Dyk and Sr Basson, and the clinical team at Karl Bremer Hospital, especially Janine, for referring participants and being such an enthusiastic collaborator.

Finally, I wish to express my deepest thanks to my family. Thank you, Teresa and Sammy, for being so patient and good-natured. It is such a blessing to be able to smile and laugh every day: thank you for all the joy you bring to me every day. I love you very much.

To my parents, Fernando, Maria Cristina, Laubi and Christina: I am blessed to have you. Thank you for your support.

To Lourens: you inspire and strengthen me every day, with your kindness, humility, generosity and utmost integrity. Thank you for always believing in me, for sharing your passion for science, knowledge and analysis. You enrich my life in every way.

## Appendix G

### Funding

This doctoral research was supported by a scholarship for doctoral studies from the Medical Research Council of South Africa under MRC Clinician Researcher Programme.

This work was also supported by funding from the Faculty of Medicine and Health Sciences at Stellenbosch University (Early Career Grant - salary support - and Temporary Research Assistantship grant - project support), the Harry Crossley Foundation (project support), the South African National Research Foundation (Thuthuka programme funding for doctoral students - PI E Walters - project support), the South African Medical Research Council (Self-initiated Research program; PI E Walters - project support), the Tuberculosis Trials Consortium (15FED1511233, Centers for Disease Control and Prevention; PI: AC Hesselning - project support), the Foundation for Innovative New Diagnostics (FIND; PI: E Walters - project support), the International Maternal Pediatric Adolescent AIDS Clinical Trials (IMPAACT; PI: AC Hesselning - salary support.), and the International Tuberculosis Specialty Laboratory (ITBSL; PI. A. Demers - project support). Overall support for the IMPAACT Network was provided by the National Institute of Allergy and Infectious Diseases (NIAID) with co-funding from the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) and the National Institute of Mental Health (NIMH), all components of the National Institutes of Health (NIH), under Award Numbers UM1AI068632 (IMPAACT LOC), UM1AI068616

(IMPAACT SDMC) and UM1AI106716 (IMPAACT LC), and by NICHD contract number HHSN275201800001I.

My salary was also supported in part by funding from the South African National Research Foundation's SARCHI Chair in Paediatric Tuberculosis (PI: AC Hesselning).

Additional funding to support the bio-repository was received from the NIH (1R01HD090927-01A1: Direct quantitation of the circulating *M.tb*-peptidome for pediatric TB management; PIs T Hu and E. Walters) and from the Thrasher Research Fund (GENFD0001203422: Urine protein biomarkers for tuberculosis diagnosis and treatment response in children; PIs R Husson and E. Walters).