Detection of *Mycobacterium tuberculosis* complex using Xpert MTB/RIF Ultra in the stool of paediatric patients

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Abbreviations

BCG	Bacillus Calmette–Guérin vaccine
DST	Drug susceptibility test
DR-TB	Drug-resistant tuberculosis
HIV	Human Immunodeficiency Virus
ICF	Informed Consent Form
IGRA	Interferon gamma release assay
MSF	Médecins Sans Frontières/Doctors without Borders
MDR-TB	Multidrug-resistant tuberculosis
MTB	*Mycobacterium tuberculosis*
MoHS	Ministry of Health and Social Welfare
NTP	National TB programme
OCA	Operational Centre Amsterdam
PCR	Polymerase Chain Reaction
SOP	Standard Operating Procedure
RR-TB	Rifampicin resistant tuberculosis
TB	Tuberculosis
TST	Tuberculin skin test
WHO	World Health Organisation

Summary

In 2017, TB was the leading infectious cause of death globally. Since children provide the reservoir out from which future cases will develop, there are strong public health arguments for focusing on childhood TB beyond the clinical imperative to aim to detect and treat a curable disease. Difficulties in collecting clinical samples, lower rates of laboratory confirmation from specimens, diverse clinical spectrums of disease making clinical diagnosis more complex, and the under-utilisation of child-health services by remote communities are all likely to result in under-estimation of global burdens. A major barrier to improved service delivery is the difficulty in establishing an accurate TB diagnosis in children.

Estimates of the burden of TB disease in children suggest that current case finding strategies are grossly inadequate. Although improvements in the use of clinical assessments may have positive effects, wide variations in clinical syndromes, rapid progression of symptoms and non-specialist medical staff limits the feasibility of this approach more widely.

Since the release and rapid roll-out of the Xpert assay, much interest has been paid to the impact this fast and sensitive technology can have on case detection and ultimately treatment outcomes. Sadly, the lesser performance of this assay in children and in non-respiratory specimens has failed to positively affect this global challenge.

With the release of Xpert Ultra, evaluation of this more sensitive test using non-respiratory specimens is imperative to guide its future application in programmatic detection of paediatric TB. We propose a prospective laboratory diagnostic study amongst children under 15 years to evaluate the performance Xpert Ultra from stool specimens compared to Xpert Ultra from sputum specimens.

All children aged 15 years either presenting or referred with suspected pulmonary TB to the Paediatric Hospital in Dushanbe will be eligible for inclusion. Patients clinical features and diagnostic test results will be recorded and they will be asked to provide a stool specimen in addition to routine sputum specimens.

Specimens will be processed and tested using the Xpert MTB/RIF Ultra test and results provided to clinicians.

The primary outcome measure will be the sensitivity and specificity of Xpert MTB/RIF Ultra testing on stool specimens when compared with the composite “confirmed TB” definition from respiratory specimens. Secondary outcome measures will include frequency of invalid or error results, frequency of “trace” calls, and proportion of stool specimens that were insufficient.

Data will be recorded in laboratory databases and clinical results will be shared with treating clinicians. Study data will be recorded in specific password-protected electronic databases.

This study is a result of the ongoing collaboration between MSF and the MoH in Tajikistan. The National TB programme will actively participate in every stage of the study and will assist in the sharing of results with patients and their communities, as well as with other national stakeholders.

1 Introduction

1.1 Context

Tajikistan is a mountainous landlocked country in Central Asia bordered by Afghanistan, China, Kyrgyzstan, and Uzbekistan. In 2017, the World Bank reported Tajikistan to be amongst the poorest 30 countries in the world (1). Home to approximately 9 million people, over 95% of the population identify themselves as Muslim. The population structure is pyramidal, with approximately 35% represented by those younger than 14 years (Figure 1).

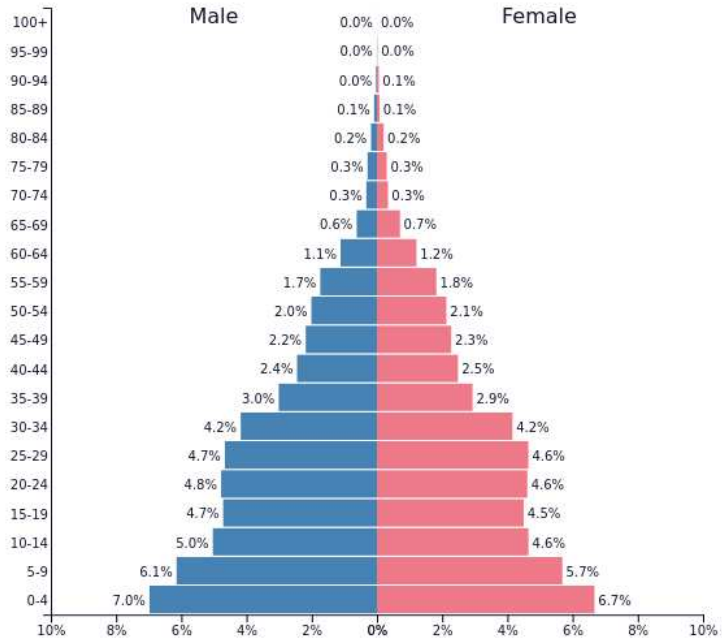

Figure 1. Population age and gender structure for Tajikistan, 2018 (2)

Literacy amongst adults is high (99%) with Tajik being the national language, and Russian frequently used for commercial purposes.

The country is divided into 65 districts encompassed by 4 administrative regions. Dushanbe, the capital, has a population of approximately 800 000.

Similarities between the Tajik healthcare system and others in the region, reflect the common recent history of the Soviet era between 1922 and 1991. Health care quality was good during this period but despite efforts from the national government, lack of resources, expertise and limited access especially in remote rural areas remain problematic. The state remains the main public funder, but significant financial support is provided by external funders.

Tajikistan is recognised by the World Health Organisation (WHO) as a country with a high-burden of multidrug-resistant tuberculosis (MDR-TB) with an estimated 1200 cases each year. While TB incidence in Central Asia (Tajikistan: 85 per 100 000) is lower than in Sub-Saharan Africa (Botswana: 326 per 100 000), very high proportions of patients diagnosed with the disease suffer with the drug resistant form (Tajikistan: 22%; Botswana: 3.6%)(3). The predominantly low HIV prevalence in Central Asia alongside the relative predominance of drug-resistant TB (DR-TB) means that experience from other regions are frequently difficult to directly translate to Tajikistan. Recent Tajik

data from the capital district, Dushanbe, shows 26.7% of newly diagnosed and 40.2% of retreatment cases suffering with MDR-TB.

1.2 Global paediatric TB

In 2017, TB was the leading infectious cause of death globally. Since TB is largely a disease of poverty, sufferers frequently live in poor communities with little access to health services.

Children suffering with TB suffer even greater levels of neglect, partly through the perception that they are rarely infectious and rarely develop severe disease, and partly because child health has received limited attention in many countries (4). Since children provide the reservoir out of which future cases will develop, there are strong public health arguments for focusing on childhood TB beyond the clinical imperative to aim to detect and treat a curable disease.

In recent years, interest in paediatric TB has dramatically increased. In 2012, the WHO included an estimate of childhood TB in their annual global TB report for the first time (5) and launched the Child TB roadmap emphasising the need to address persistent policy-practice gaps (6). In parallel, funding for research on child-friendly paediatric drug formulations and pharmacokinetic studies in children has been increasing.

Despite this progress, there are a number of challenges to estimating the burden of TB in children. Most estimates use case notification as a starting point, despite the fact that in many countries children starting TB treatment are not notified to national authorities. Difficulties in collecting clinical samples, lower rates of laboratory confirmation from specimens, diverse clinical spectrums of disease making clinical diagnosis more complex, and the under-utilisation of child-health services by remote communities are all likely to result in under-estimation of global burdens.

In 2016, based on national reporting, the WHO estimated that 550 000 incident cases of TB occurred in children with 201 000 dying from their disease. This corresponds to 10% of all incident cases occurring in children. Case detection and notification amongst children was lower than for adults, with younger children affected the most (figure 2).

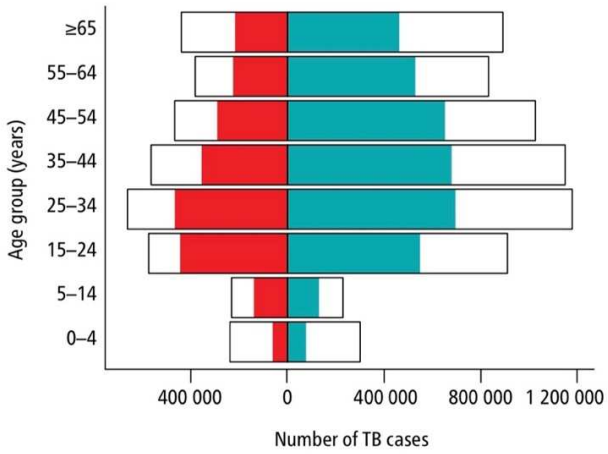

Figure 2. Global estimates of TB incidence and case notifications disaggregated by age and sex (female in red; male in green), 2016 (7)

Children suffering with DR-TB are also under-represented in national and global figures. The WHO estimates that in 2016 there were 600 000 incident cases of MDR-TB globally with approximately 240 000 deaths (7). While they do not provide an estimate amongst children, modelling studies have approximated this figure to be around 32 000 (8). Challenges associated with laboratory confirmation of disease in children are exacerbated amongst those with DR-TB since many national TB programmes require laboratory verification of drug resistance prior to starting second line treatment.

Tajik national data shows that paediatric DR-TB cases represent 6.5% of total DR-TB cases in the country, a lower proportion than that seen in all forms of TB (11%).

1.3 Laboratory diagnostics for paediatric TB

Following infection, young children have a high risk of progression to TB disease. Approximately 30-40% of infants infected will progress to intrathoracic TB and 10-20% will develop disseminated disease. Generally, risk of progression declines as age increases in children (9).

A major barrier to improved service delivery is the difficulty in establishing an accurate TB diagnosis in children. While clinical findings can be variable, especially amongst younger children, persistent cough, weight loss or failure to thrive, persistent unexplained fever, persistent unexplained lethargy or reduced playfulness, or the presence of any of the following in neonates: pneumonia, unexplained hepatosplenomegaly or sepsis-like illness, have been proposed by expert groups as one component of clinical case definitions of intrathoracic TB disease (10).

A consensus classification of the certainty of TB disease categorises confidence into “confirmed”, “probable” and “possible” as outlined in Table 1 (11).

Table 1. Classification of certainty of diagnosis of TB in children

Recommended category	**Definition**
Confirmed TB disease	At least 1 sign or symptom of TB disease **and** microbiological confirmation of *M.tuberculosis*
Probable TB disease	At least 1 sign or symptom of TB disease **and** a chest x-ray consistent with intra-thoracic disease **and** presence of one of the following: a) clinical response to TB treatment, b) exposure to a source case with TB disease, c) immunological evidence of TB infection
Possible TB disease	At least 1 sign or symptom of TB disease **and either** a chest x-ray consistent with intra-thoracic disease **or** presence of one of the following: a) clinical response to TB treatment, b) exposure to a source case with TB disease, c) immunological evidence of TB infection

Laboratory diagnostics for TB infection and disease can be divided into immunological and bacteriological. The tuberculin skin test (TST) and IFN-γ release assays (IGRA) both detect previous exposure to TB and stimulate a measurable response through reactivation of immunological

pathways. Whilst TST is relatively cheap, it does require administration followed by delayed measurement. False negative results are common in children and HIV co-infected patients with up to 20% of culture confirmed children failing to register a positive result. False positive results can occur following BCG vaccination. In contrast to TST, IGRAs require a single blood sample. However, they are more expensive and require greater expertise in the laboratory. The use of positive and negative controls helps to validate individual results. IGRAs have higher specificity than TST for TB infection amongst children (85-95% vs 45-60%), particularly in low incidence settings and among BCG vaccinated subjects (12). However, IGRAs also perform poorly in immunocompromised patients and children with severe TB disease and cannot differentiate infection from disease.

Molecular detection of TB from clinical specimens in developing countries is predominantly performed by Xpert MTB/RIF (Xpert). It utilises the GeneXpert platform, a self-contained cartridge-based system that automates the necessary steps of the PCR process. The test performs particularly well in smear-positive sputum with pooled sensitivity and specificity reported as 98% and 99% respectively (13). In children, Xpert performs worse with pooled sensitivity reported as 62% when compared to culture. However, it remained 35-45% more sensitive than direct smear microscopy (14). Due to the paucibacillary nature of TB in children, culture negative clinically diagnosed cases are common. When clinical diagnosis of TB is used as the reference standard in children, Xpert sensitivity drops to 4% for sputum and 15% for gastric aspirate specimens (15). A second component of the Xpert assay is the detection of mutations in the *M.tuberculosis rpoB* gene from clinical specimens. Mutations in this gene are strongly associated with rifampicin resistance and MDR-TB. Pooled sensitivity and specificity amongst adults are 95% and 98% respectively (13).

Xpert has been evaluated on non-respiratory specimens in children. Since young children swallow respiratory secretions and sputum, specimens from the gastro-intestinal tract have been of particular interest to detect TB in children. Gastric aspiration requires the passing of a nasogastric tube and suction of stomach contents prior to neutralisation and laboratory testing. Since stool can be collected non-invasively, it has been of specific interest as a specimen for molecular and culture-based detection. Studies of varying sizes with differing comparator groups have yielded a wide range of sensitivity estimates (32-83%) but all have confirmed high levels of specificity (16-19).

Mycobacterial culture remains the WHO-recommended gold standard for diagnosis of TB disease. Isolation of organisms is important for definitive diagnosis as well as for phenotypic drug susceptibility testing (DST). Sensitivity of culture for detection of TB disease in children is lower than amongst adults due to the pauci-bacillary nature of paediatric disease. The long duration required for culture (up to 6 weeks) and the rapid progression of disease in younger children frequently necessitates treatment initiation prior to positive results. Culture confirmation of childhood TB typically occurs in <40% of cases (20). Direct smear microscopy is positive in <20% of gastric aspirate and induced sputum specimens (21). Mycobacterial culture requires high levels of expertise and well-resourced specialist laboratories.

1.4 Xpert MTB/RIF Ultra

The Xpert assay was developed and endorsed by the WHO in 2010 to improve TB and rifampicin resistance detection. Automating many of the steps required to process clinical samples, and improving the sensitivity of detection of both MTB and genetic mutations resulting in rifampicin resistance, yielded a platform that could provide highly accurate results in approximately 2 hours from laboratories with limited expertise. Since the presence of rifampicin resistance is highly predictive of MDR-TB, this dramatically improved the quality and speed of determining resistance to

this key drug. Pooled data show overall sensitivity and specificity of approximately 89% and 98% respectively (13). Uptake of the platform developed by Cepheid has been rapid due both to the performance of the MTB/RIF assay, but also the modular nature of this platform. Diagnostic Xpert cartridges are available for HIV, Hepatitis C, sexually transmitted infections, influenza and drug resistance amongst bacterial pathogens. Between 2010 and 2016 over 23 million Xpert tests have been procured by 130 countries (22).

Xpert MTB/RIF does have limitations in performance particularly on smear-negative sputum (pooled sensitivity 67% (13)), and those suffering with HIV co-infection (43% sensitivity (23)). Also, despite the impressive global scale-up of the test, clinical impact has been lower than expected (24). Reduced sensitivity in some extrapulmonary samples which are known to contain fewer bacteria also disproportionally affects children and HIV co-infected patients who suffer more frequently with this manifestation of the disease.

The Xpert MTB/RIF Ultra assay (Xpert Ultra) was developed to overcome the limitations of the Xpert assay. Improvements in molecular technology alongside assay chemistry and cartridge redesign have resulted in an approximately 1-log improvement in the lower level of detection of Xpert Ultra compared with Xpert (25). Clinical studies have shown improvements in sensitivity from 46% to 63% in smear-negative and culture positive patients, 77% to 90% amongst culture positive HIV co-infected patients (26), and from 45% to 95% amongst HIV co-infected patients suspected of having tuberculous meningitis (27). Assessment of the performance of Xpert Ultra on extra-pulmonary specimens is limited. One study of 82 culture positive specimens showed Xpert Ultra to be 76% sensitive, while amongst 4 culture positive stool specimens sensitivity was 80% (28).

One consequence of improvements in sensitivity of Xpert Ultra were slight reductions in specificity especially amongst patients with previous treatment history. To highlight the potential for false positive results at very low levels of detection, Xpert Ultra provides a “trace” call. Early implementation reports of Xpert Ultra suggest that the frequency of trace call results is higher amongst previously treated patients. In Central Asia, where prior TB treatment is more common amongst adults, the positive predictive value of Xpert Ultra is likely to fall. In response to concerns regarding interpretation, the Global Laboratory Initiative has recommended that “among persons with HIV, children and extra-pulmonary specimens ‘trace calls’ should be considered to be true positive results” (29).

In 2017, owing to these improvements in performance, the WHO recommended Xpert Ultra as a replacement for Xpert as a screening test for detection of TB.

1.5 MSF in Tajikistan

Médecins Sans Frontières has been working with the Ministry of Health and Social Welfare in Tajikistan since 2011. From the outset, the collaboration in Dushanbe has been targeted towards paediatric DR-TB because of a mutual recognition of under-detection. After initially focusing on treatment provision through national TB programme (NTP) models of care, strengthening of the collaboration since 2013 has resulted in bolder ambitions. Focusing on 4 areas around Dushanbe (Rudaki, Hissor, Vahdat and Dushanbe itself), the project has, in recent years, introduced sputum induction services, increased access to DR-TB treatment and child-friendly formulations, introduced ambulatory care from day 1 and family-assisted observed therapy, supported the NTP in contact tracing, implemented the short regimen for MDR-TB and increased the use of new DR-TB drugs in children.

Improving the diagnosis of children with TB has been an important aspect of the collaboration given the technical challenges outlined. Education of family doctors, support of TB doctors and incorporation of Xpert and sputum induction into laboratory testing have all contributed to increases in the detection of cases. Since 2016, support of household contact tracing in Dushanbe increased paediatric TB investigation and case detection through greater identification of symptoms and referral for sputum examination.

2 Study rationale

Estimates of the burden of TB disease in children suggest that current case finding strategies are grossly inadequate. Although improvements in the use of clinical assessments may have positive effects, wide variations in clinical syndromes, rapid progression of symptoms and non-specialist medical staff limits the feasibility of this approach more widely.

Since the release and rapid roll-out of the Xpert assay, much interest has been paid to the impact this fast and sensitive technology can have on case detection and ultimately treatment outcomes. Sadly, the lesser performance of this assay in children and in non-respiratory specimens has failed to positively affect this global challenge.

With the release of Xpert Ultra, evaluation of this more sensitive test using non-respiratory specimens is imperative to guide its future application in programmatic detection of paediatric TB.

3 Hypothesis

Amongst children aged under 15 years with confirmed TB disease, sensitivity of Xpert MTB/RIF Ultra testing of stool specimens will be >60%.

4 Research objectives

4.1 Primary objective

1. To describe the performance of Xpert MTB/RIF Ultra assay for *M.tuberculosis* detection on fresh stool specimens amongst children under 15 years of age with confirmed TB disease.

4.2 Secondary objective

1. To describe the performance of Xpert MTB/RIF Ultra assay for detection of *M.tuberculosis* on fresh stool specimens from children with confirmed, probable and possible TB.
2. To describe the performance of Xpert MTB/RIF Ultra assay for detection of rifampicin resistance on fresh stool specimens from children with confirmed, probable and possible RR-TB.
3. To describe the practical feasibility of performing Xpert MTB/RIF Ultra on stool specimens using a standardised laboratory protocol.
4. To describe the frequency of the “trace call” from the Xpert MTB/RIF Ultra assay using stool or sputum samples.

5. To describe treatment prescription practices 6 weeks following Xpert MTB/RIF Ultra assay testing.

5 Patients and Methodology

5.1 Study design

We will conduct a prospective laboratory diagnostic study in one facility in Dushanbe, Tajikistan.

5.2 Study sites

This study will be conducted at the Dushanbe PD hospital, a referral centre for paediatric TB and sputum induction services. Sputum induction has been implemented at this site since 2013. Over 1000 sputum induction procedures are performed annually by 6 trained nurses. Specimens are testing using Xpert at an on-site laboratory by 2 trained laboratory technicians. Approximately 65 children under 15 are referred for diagnostic sputum induction each month, all of whom would be eligible for inclusion in this study. On average, yield from sputum induction and Xpert testing is 6.5%.

5.3 Partnership/study coordination

The study will be conducted in a partnership between the Ministry of Health of Tajikistan and Médecins Sans Frontières/Doctors without Borders (MSF) – Operational Center Amsterdam (OCA). The Tajik National TB Programme and the Ministry of Health have actively participated in protocol development and will support study implementation, interpretation and publication of results. MSF OCA will act as the sponsor of the study.

The study will be implemented by a medical team consisting of nurses and laboratory technicians currently working on site. Data collection and analysis will be supported by a data entry operator and epidemiologist. A laboratory supervisor and the nurse supervisor will be responsible for training, implementation and monitoring including the drafting of regular monthly progress reports. They will also be responsible for ensuring good collaboration between the MSF and MoHS staff working at the laboratory and in the sputum induction facilities. Existing nurses will ensure proper information is given to patients/guardians, consent is obtained prior to sample collection, all required data are collected and recorded in the appropriate forms and samples are collected according to the study standard operation procedures (SOPs). Laboratory technicians at the site will be responsible for sample processing and analysis according to the study SOPs, recording results in designated forms and databases and giving them to respective medical doctors. The study team will ensure correct implementation and follow-up of the study procedures and will discuss any challenges/difficulties with the primary investigator.

5.4 Training

All MSF medical staff and selected MoHS staff (nurse, laboratory technician) working at the study site will be trained prior the initiation of the study by selected international staff (Nursing activity manager, Nurse supervisor, Laboratory manager). Training will focus on:

- basic information about the study for all MSF medical staff and medical staff in PD hospital;
- study objectives, inclusion and exclusion criteria, benefits and risks for the participants for recruiting MSF nurses working in outreach and selected MoHS staff (MDs, nurses) working at referral points in Dushanbe;

- obtaining voluntary consent from the patient for MSF nurse supervisor, sputum induction nurse;
- sputum induction procedure refreshment for sputum induction nurse;
- data collection, storage, patient confidentiality for sputum induction nurse, laboratory technicians, data manager;
- laboratory procedures for safe sample handling, sample analysis, data collection and QC analysis for laboratory technicians.

5.5 Study participants and sample size

Inclusion criteria

All children aged under 15 years either presenting or referred with suspected pulmonary TB to the Paediatric Hospital in Dushanbe will be eligible for inclusion. No lower age limit for inclusion will be used in this study.

Exclusion criteria

- Children who have received > 24 hours of TB treatment (excluding isoniazid prophylaxis therapy) within the preceding 30 days,
- refuse to participate in the study, or
- where collection of a sputum and/or stool sample is not possible within 3 days of each other will be excluded.

Liquid stool will be accepted by the laboratory for processing and the presence diarrhoea will not be an exclusion criteria for the study.

To assist in measurement of bias, children whose guardians refuse for them to participate in the study will be described by frequency, age and gender.

Determination of sample size

The primary analysis will describe the performance of Xpert Ultra from stool in subjects with confirmed TB (i.e. with laboratory confirmation of TB).

Table 2. Sample size estimation with a variety of assumptions

Expected sensitivity	Precision			
	0.05	0.07	0.1	0.12
0.5	385	196	97	67
0.55	381	195	96	66
0.6	369	189	93	64
0.65	350	179	88	61
0.7	323	165	81	56

If sensitivity is estimated to be 60% with precision of 0.12, then 64 subjects diagnosed with confirmed TB will be required. A conservative estimate of detection amongst children undergoing sputum induction would be 5% which suggests 1300 children would need to be included. If we assume that 10% of parents will refuse participation, 1430 children and their parents would need to

be approached about the study, which at the current rate of sputum induction referral would take approximately 22 months.

5.6 Study endpoints

The study's primary endpoint will be the result of stool Xpert Ultra testing from children with "confirmed TB" as defined in section 5.7 below. The classification of certainty of TB disease will be used to identify children with confirmed TB disease. Confirmed TB disease will serve as a gold-standard, such that the sensitivity, specificity, positive and negative predictive values for Xpert Ultra on stool specimens can be reported.

Possible results for the **primary endpoint** are:

- MTB detected (high, medium, low or very low),
- MTB detected (trace),
- MTB not detected,
- Invalid, error or not result

Secondary endpoints will include:

- Rifampicin resistance detection result from Xpert Ultra (secondary objective 1)
    - Rifampicin resistance detected, not detected, indeterminate
- Qualitative and operational aspects of Xpert Ultra using stool implementation including any additional need for human resources or training, additional laboratory consumables, specimen transportation requirements and result dissemination (secondary objective 3).
    - Frequency of receipt of insufficient or unusable stool specimens
- TB treatment prescription 6 weeks following Xpert Ultra testing on stool to evaluate
    - Possible endpoints will be:
    - No TB treatment started,
    - DS-TB treatment started,
    - Treatment for RR-TB started
    - Unknown

5.7 Case definitions

Participants risk of TB will be classified based on consensus definitions using clinical, radiological and laboratory assessments (Table 1) (11).

The following clinical signs and symptoms will be used to categorise risk of TB disease:

One or more of the following:

- Cough ≥ 2 weeks
- Unexplained fever ≥ 1 week, or
- Poor growth or weight loss over the preceding 3 months

For children <1 year old, the following will also be acceptable:

- Pneumonia
- Unexplained hepatosplenomegaly
- Sepsis-like illness

Microbiological confirmation of TB disease constitutes:

One or more of the following from respiratory specimens:

- Positive smear microscopy results, or
- MTB detected Xpert MTB/RIF or Xpert MTB/RIF Ultra results, or
- M. tuberculosis complex detected in Hain MTBDRplus results, or
- Positive for M. tuberculosis complex in MGIT or LJ culture

Microbiological confirmation of rifampicin resistance

One or more of the following detecting rifampicin resistance from respiratory specimens:

- Xpert MTB/RIF or Xpert MTB/RIF Ultra, or
- Hain MTBDRplus
- Phenotypic DST from MGIT or LJ

Risk factors for rifampicin resistance

One or more of the following:

- TB treatment failure, or
- Death during TB treatment, or
- Loss to follow-up or poor adherence record during TB treatment, or
- Contact with a person known to suffer with RR-TB

Table 3. Classification of risk groups for TB disease and rifampicin resistant TB disease

Certainty of TB diagnosis	Confirmed TB disease	At least 1 sign or symptom of TB disease and microbiological confirmation of *M.tuberculosis*
	Probable TB disease	At least 1 sign or symptom of TB disease, and chest x-ray consistant with intra-thoracic TB disease, and 1 of the following: (a) a positive clinical response to TB treatment, or (b) documented exposure to a source case with TB disease.
	Possible TB disease	At least 1 sign or symptom of TB disease, and either (a) a chest x-ray consistent with intra-thoracic TB disease, or (b) a positive clinical response to TB treatment or documented exposure to a source case.
Certainty of rifampicin-resistant (RR) TB	Confirmed RR TB	As for confirmed TB disease above with additional demonstration of rifampicin resistance through genotypic or phenotypic tests
	Probable RR TB	As for probable TB disease above with additional documented RR-TB contact
	Possible RR TB	As for probable TB disease above with either (a) contact of a source case of TB with risk factors

		for drug resistance, or (b) failure of first-line TB treatment

6 Study procedures

6.1 Informed consent

All children under 15 years old suspected of suffering with TB with their parent or legal guardian will be invited to participate in the study when they attend for diagnostic sample submission at the Paediatric Hospital in Dushanbe. The informed consent process will involve a thorough explanation of the study, the opportunity to ask questions and have them answered, and the signing of an informed consent form. Children who are deemed competent will be asked to give written consent alongside their guardian or parent, while those who have sufficient understanding will be asked to give their assent according to local practice.

6.2 Sample collection

One stool sample will be requested alongside the routine collection of either expectorated sputum or induced-sputum specimens from each subject. As per local policy, two respiratory specimens will be collected; one for molecular testing and a second for mycobacterial culture, when required. Stool and sputum samples will be collected within 3 days of each other. Patients not able to produce all the requested samples will be asked to make further attempts during the following days, but not after one week. If sufficient specimen is obtained within 3 days, this will be processed even if not all the requested number of specimens are obtained. Patients not able to produce at least one sputum specimen and one stool sample will not be included in the study. These procedures will be conducted according to local guidelines and laboratory practices.

6.3 Laboratory procedures

The sputum sample will be processed using the Xpert MTB/RIF Ultra cartridge and smear microscopy in Dushanbe TB Paediatric Department lab. Positive specimens are routinely referred to the National Reference Laboratory for culture and phenotypic DST.

Approximately 0.6 grams (+/- 0.2 grams) of the stool sample will be weighed and processed according to the protocol described in Annex 5. In summary, 2 ml of the stool processing buffer (SPB), containing AL buffer (Qiagen, Valencia, CA) and 10% Poly-vinyl-pyrrolidone (Sigma Aldrich, St. Louis, MO), 2 ml of Xpert MTB/RIF sample reagent (SR) (Cepheid), and 3 mm glass beads (Fisher Scientific, Pittsburgh, PA) will be added to the stool. After a short mix and 30 min incubation, the mixture will be passed through a syringe filter (fitted with glass wool to capture the stool debris) into a clean collection vial. Two ml of this filtrate will be loaded into the sample chamber of an Xpert MTB/RIF Ultra assay cartridge. Subsequent sample processing and PCR will be performed in accordance with the manufacturer's recommendations using GeneXpert instrument and the analysis will be done by the GxDx software version 4.8.

Results from stool testing will be communicated to referring clinicians where they may influence patient management. This will be done by telephone with follow-up paper reports being disseminated as per local practice. In patients where stool samples yield positive results either through detection of TB or rifampicin resistance, and sputum tests are negative, stool results will be communicated to referring clinicians with advice to speak to local TB specialists about interpretation

and appropriate clinical management. Where stools results are concordant with sputum results, no additional information will be provided to referring clinicians.

6.4 Sample handling and storage

Respiratory specimens will be collected at the clinic and samples will be kept between 2 – 8°C until processed. Processing will be done within the 24 hours and not more than 72 hours after collection.

A sterile stool container and instructions will be provided to the parent/guardian for specimen collection and handling (Annex 4). Once collected, the parent/guardian must bring it to laboratory within 12 hours since recommended storage temperatures for fresh stool samples are between 2 – 8°C. If no refrigerator is available, sample collection must be done in the morning on the day the parent/guardian is visiting the clinic. Following delivery to the laboratory, the stool specimen will be kept in a cool dry place and processed within 2 hours. Stool samples can be frozen between -20°C and -15°C if processing cannot occur immediately.

Samples obtained in this study will adhere to all local laboratory procedures governing the storage of biological specimens. Samples will not require additional storage measures for the purposes of the study and will be discarded as per local policy.

No further research will be conducted on any specimens obtained for the study without prior ethical review board and local Ministry of Health approval.

6.5 Data collection, storage and retention

Standardised data collection forms will be used. Subject data will be collected at the time of study enrolment while laboratory data will be retrieved following Xpert MTB/RIF Ultra testing of specimens. End of study follow-up data will be collected from centralised TB registers and family clinic records.

All hard copies of data will be securely stored and all computerised information will be password protected. Data will be recorded in a dedicated Excel database. Only data relevant to this study will be recorded. Merging of data for statistical analysis will be conducted using Stata or R. Backups of data will be generated regularly and stored by the in-country data entry supervisor, and the primary investigator. Access to study data will be restricted to investigators involved in data management and analysis. Following study completion, all consent forms and other data will be held in a secure location in MSF UK (Manson Unit) for 5 years, after which it will be inactively retained off-site for a further 15 years and then destroyed as per MSF UK Record Retention Policy.

6.6 Statistical analysis

Sensitivity, specificity, negative predictive value, and positive predictive value will be calculated by defining true or false positive and true or false negative against the reference standard. The proportion testing indeterminate will be reported.

Distribution of continuous variables will be assessed for normality, and where this is present, will be described using means and standard errors. Medians and interquartile ranges will be used if not normally distributed. Categorical variables will be summarised as counts and percentages. Comparisons of categorical variables across groups will use Chi-squared or Fisher's exact test. Comparisons of continuous variables across groups will use the t-test or Wilcoxon rank sum test depending on data distribution. All estimates will be presented with respective 95% confidence intervals. Concordance between diagnostic methods will be evaluated with McNemar's test and

Receiver Operating Characteristic (ROC) curve analysis. Following repetition in the laboratory, invalid tests (e.g. Xpert Ultra error) will be excluded from the primary analysis.

A comprehensive statistical analysis plan will be developed as part of the SOPs for the study.

Statistical analyses will be performed using standard statistical software such as Stata 14 (Stata Corporation, College Station, Texas, USA) and R (R Foundation for Statistical Computing, Vienna, Austria).

6.7 Study limitations

The generalisability of the study findings will be limited by the single-site approach being used. While test performance is dependent on the study population, secondary objectives relating to the practical feasibility, prescription practices and frequency of "trace calls" are likely to be most affected. Sequential inclusion of eligible participants will serve to limit potential selection bias. Generalisation of results will be possible to other urban settings in Eastern Europe and Central Asia where TB healthcare services retain many similarities from their shared Soviet history.

The reference standard used in this study will be "confirmed TB disease" as set out in Table 1. Referral of specimens for mycobacterial culture will be restricted to those with positive smear or Xpert MTB/RIF Ultra results as per local practice. This two-step process may reduce the sensitivity of TB detection by the reference standard possibly resulting a greater proportion of participants being included in secondary analyses.

7 Ethical considerations

7.1 Ethical committee

This study will be conducted following approval of the ethical committee of Médecins sans Frontières and the written approval of the Tajikistan National Tuberculosis Programme. Submission of prospective laboratory diagnostic studies for approval by the Tajik ethical review board is not required according to local practice.

7.2 Informed consent process

Informed consent is a process where information is presented to enable eligible subjects and their parents or guardians to voluntarily decide whether or not to participate in a research study. It is an ongoing-process between the subject and the researchers which starts prior to participation and ends when the subject's participation in the study ends. Essential information that will be explained by a trained health worker will include the study's purpose, the duration, additional laboratory procedures being performed, any risks or benefits to the subject or others in their community, and how their specimens will be handled and stored. The subject will have the opportunity to ask questions and have them answered.

All eligible subjects and their parent or legal guardian should be offered the opportunity to participate in the study. The staff who perform sample collection procedures will approach eligible parents or guardians regarding inclusion.

Potential risks from participation will be communicated. Parents or legal guardians will be able to ask questions and have these answered prior to deciding on participation. They will be asked to sign and date an informed consent form (ICF) (Annex 2) prior to inclusion and data collection.

The ICF will be clear and simple, and written in the parent or legal guardian's language to facilitate comprehension. If the patient is unable to read, a relative, or an impartial witness should be present during the informed consent discussion. In this case, the parent or legal guardian must give consent orally, and if capable of doing so, complete, sign (or thumbprint) and personally date the information and ICF. The witness will then complete, sign and date the form together with the investigator.

The right to withhold consent at any time during the study and the right to the same quality healthcare regardless of involvement in the study will be clearly explained in the ICF and during the consent process.

The parent or legal guardian will receive a copy of the completed ICF. They may withdraw consent at any time throughout the course of the study without any impact on their treatment or follow-up as part of the normal clinical practice in the Tajikistan.

Particular attention will be taken to ensure the process is adapted and appropriate to the age of the participant, including adapted information sheets.

Additional local requirements for informed consent to perform sputum induction will be followed by study staff.

7.3 Potential risks

The main burden to subjects involved in the study will be additional time required to provide one stool specimen within 24 hours of sputum induction. This will be explained to subjects and their caregiver during the informed consent process.

Individual level risks

There is a risk of loss of confidentiality in the collection of non-routine clinical data from subjects and the involvement of study staff who may not have been involved in routine care. Principles of confidentiality and data protection will be reinforced by the study team alongside reminders of the importance of patient confidentiality in the working contract of all healthcare workers.

When subjects are offered the chance to participate in a study by healthcare staff, there is a risk of conflict of interest. By ensuring that the informed consent process is conducted at the time of specimen collection, the subject's physician will only provide background information, while an external practitioner will answer questions and facilitate the consent process. Additionally, adding the name and contact details of a study nurse to the information leaflet and consent form will allow the subject to access information independent of their healthcare provider.

A small risk of bronchospasm is associated with sputum induction due to the need for inhalation of hypertonic saline to promote coughing. The performance of this procedure by trained staff with appropriate pre-test clinical checks and immediate availability of correct medical equipment serves to reduce the risk of adverse events.

Collection of stool specimens pose no risk to participants or their relatives.

Since individual risks to children are small, reporting of adverse events and setting up of an external data safety monitoring board are not felt necessary for this study.

Programme level risks

There is a risk that request to participate in a research study may adversely affect the relationship between the subject and the health care provider. Since few prospective studies in TB have been conducted in Tajikistan, attitudes towards research are uncertain. Explanation and information relating to the study will be provided in Tajik or Russian and opportunities to ask questions will be available prior to providing consent. The option of withdrawal from the study at any time and the provision of routine care regardless of study involvement will be fully explained to all subjects.

7.4 Potential benefits

Individual level benefits

Individual participants may benefit from participation in the study if Xpert Ultra from stool detects TB where other specimens remain negative. Although final treatment decisions will be taken by clinicians outside of the study team, the very high specificity of Xpert Ultra will likely result in TB treatment being started.

Community level benefits

Improved detection of children suffering with TB may result in improvements of community case detection through routine household contact tracing activities conducted in Dushanbe by MSF and the NTP. Households of children found to be suffering with TB are routinely followed up by local nurses to identify those requiring further investigation and to provide health promotion and information relating to TB.

Supporting policy updates

The results and conclusions from this study will help to influence an often neglected aspect of TB management – the diagnosis of disease in children. Reporting of Xpert Ultra performance on stool specimens raises the challenges of diagnosing children with TB while also providing real-world results when new tests are developed. Describing the frequency of “trace” calls will help programme managers prepare for wider utilisation of Xpert Ultra through training of clinical staff.

7.5 Confidentiality and Privacy

All staff working in each study site will be trained on the importance of patient confidentiality. Patient names will be recorded in the clinical forms for the purpose of clinical follow up. Patients will be identified by a unique identification number from the outset of the study and this number will be used for all study databases and reports. Raw data exported from site electronic and paper sources will be de-identified prior to transferring for analysis. Source data will only be accessible to designated clinical and research key personnel and national authorities.

The privacy of patients will be protected as per routine practice in the study site. Attendance to the sputum induction facility, while potentially stigmatising, has proven acceptable to the local community. Potential participants will be approached by their treating doctor with information regarding the study when they are referred for further diagnostic evaluation. Additional information beyond that required by the study will not be requested or collected.

7.6 Sharing findings with participants

The findings of the study will be shared and discussed with participants, MoHS and MSF teams through a short presentation and a summary of findings booklet. This will be prepared in

collaboration with all co-investigators and will be shared and approved by mission and MoHS teams prior to dissemination to ensure the content and language are appropriate for the audience.

8 Budget

Costs related to the study have been incorporated into the MSF operational budget. All consumables and diagnostic tests will be available through standard MSF procurement and supply mechanisms. Additional human resources for this study are not anticipated, and costs of dissemination of results and final reports will be incorporated into the MSF OCA medical department budget.

Table 4. Budget estimation (€)

Budget lines	Budget (€)	Comment
Laboratory equipment	58000	Xpert Ultra cartridges, GenX computer, scale, Vortex mixer, Buffer and other consumables required for DNA extraction
Laboratory: consumables and small material	5000	Tubes, transfer pipettes, glass beads, filters, stool containers,staining by Auramine
Other Medical: consumables and small material	200	syringes for stool manipulation
Supplies, stationery and other consumables	1200	single use knife for stool preparation, printing of consent, brochures, education material, organisation of training
HR	3000	Nurse to partially support data collection: 50% of the contract
Dissemination of results	2500	Editorial support, publication costs, conference fees

9 Timeline

A preliminary timeframe is outlined below. Adjustments will be made following the ethical review process based on the following estimations:

Ethical review: 1.5 months

Inclusion and Data collection: 18 months

Data analysis and write up: 2 months

Dissemination and follow up: 3 months

10 Dissemination of study results

The findings of this diagnostic study will be disseminated to investigators, sites, study participants and their communities through the development of summary documents and presentations. Further dissemination to key stakeholders will occur to allow the results to inform local, national, regional and global policy towards the diagnosis of TB amongst children. The research team will prepare

abstracts, presentations and manuscripts for publication in peer-reviewed journals to ensure knowledge gained is accessible to other investigators in this field. Scientific reports will be written according to STARD 2015 standards (30).

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12 Annexes

12.1 Annex 1. Participant information leaflet

Detection of Mycobacterium tuberculosis complex using Xpert MTB/RIF Ultra in the stool of paediatric patients

Ministry of Health and Social Protection and Médecins sans Frontières – Operational Center Amsterdam (MSF-OCA)

Information leaflet

Médecins sans Frontières (MSF) jointly with the Ministry of Health (MoHS) and National Tuberculosis Program is providing health care for children and their family who are suffering from tuberculosis (TB) in Dushanbe and surrounding areas.

Tests for TB are usually done on sputum. The same test can be used on stool, which is often easier for children to provide. We want to compare the results from using the test on sputum and on stool from the same children to check whether testing both samples is better than only testing one.

Your child has been referred to this facility to undergo sputum induction as your doctor suspects your child may have TB. As a result, your child is eligible to participate in this study, so we would like your permission to test his/her sputum and stool samples.

- Sputum induction for collection of sputum is part of the normal process for testing for TB in children in our program and what your doctor has referred your child for. Whether the child participates in the study or not, this will be performed with your permission. The nurse will explain in more detail how this procedure works.
- If you decide that your child can participate in the study, we ask you to bring a stool sample collected according to the instructions in the brochure to the laboratory tomorrow when you come to collect the result of the sputum test.
- The results of sputum will be given to you to pass to your referring doctor who will decide whether tuberculosis treatment is needed or not. The result from the stool will be given to your referring doctor only when the result may support management decisions.
- If by either method we confirm the child has TB they will be accepted into either the MoHS or joint MoHS/MSF program and be treated free of charge for their disease.
- We will call you six weeks after the test results to ask you about any TB treatment your child may be receiving. We will also ask your permission to access your child's health records if they have started TB treatment.

Information collected will be kept confidential and used only for the purpose of the study and for deciding on whether your child needs treatment. Results of the tests will be recorded electronically in a protected database. Anonymous results will be shared with MoHS of Tajikistan and outside the country through scientific reports, or conference presentation – this will be a summary combining information from all participants, so results from individual children will not be shown.

Your participation in this study is completely voluntary and you are free to withdraw your child at any time. Neither you, or your child will receive any direct monetary benefit such as food or payment as reward for participating in this study.

If you have any questions, feel free to ask us, we are happy to answer all your questions. If you have questions later, please contact our nursing supervisor:

Valyoat Gafurova MSF, Bukhoro Street 5-3, Dushanbe, 934440503.

12.2 Annex 2. Participant Informed Consent Form

Detection of Mycobacterium tuberculosis complex using Xpert MTB/RIF Ultra in the stool of paediatric patients

Ministry of Health and Social Protection and Médecins sans Frontières – Operational Center Amsterdam (MSF-OCA)

Participant informed consent

MSF and the Ministry of Health and Social Protection invite you and your child to participate in a study to evaluate the performance of Xpert Ultra on stool specimens in the detection of tuberculosis. Please read the following information so that you can make an informed decision about whether or not participate in the study. If you would prefer, this document can be read to you.

Tuberculosis (TB)

Your doctor has referred your child to this testing clinic because they believe that your child may be suffering with tuberculosis – an infectious disease typically transmitted through the air. While tuberculosis in children can be effectively treated, confirming the diagnosis through laboratory tests can be difficult because of challenges in collecting specimens for testing and laboratory tests which have limited performance on these specimens.

Purpose of study

A new, improved version of a test for TB is about to be released in Tajikistan by the MoHS. It has been shown in other countries to provide fast, accurate results for adults providing sputum. Since younger children cannot easily provide sputum specimens, the aim of this study is to evaluate whether the test can be used reliably on stool provided by children. We will do this by testing sputum obtained via induced sputum collection and comparing results from testing stool provided by your child. All children referred to this induced sputum centre will be eligible to participate in this study.

Methods

- If you agree to your child participating in this study, you will be asked some routine questions by the nurse in this clinic. These will relate to your child's symptoms, the presence of any unwell household members, and whether your child has had an x-ray or tuberculin skin test (TST) in recent weeks. The nurse will then explain the sputum induction procedure. This is part of the normal process for testing for TB and the reason you were referred by your doctor, so will happen even if you don't participate in the study. A stool sample will be required from your child within 3 days of sputum induction. A specimen container and an instruction leaflet will be given to you by the nurse. You will be asked to return the specimen within the container when you collect the results of your child's sputum induction test. If the test from the stool provides more information than the sputum result, this will be phoned to your doctor. We will call you six weeks after the test results to ask you about any TB treatment your child may be receiving and ask your permission to access your child's health records if they have started TB treatment.

Risks

We do not anticipate any risks to you or your child from participation in this study since treatment decisions will continue to be made by your doctor and the TB consilium and the additional sample required by the study is stool that would normally be disposed.

Benefits

Participation in the study will involve an additional test being performed to detect TB in your child. The result of this test will be shared with your doctor which may help him/her in establishing the correct diagnosis.

There will be no financial benefits to you associated with participation in this study.

Participant rights and confidentiality

You have the right to withhold consent at any moment during the study even after initially agreeing for your child to participate. Your decision at any time will be respected and this will not affect the quality of care you will receive.

Your participation in the study will be managed confidentially with information only being passed to your doctor if necessitated by important stool results. All information, including test results, relating to you or your child will be stored in a secure and safe manner. The sputum and stool specimens from your child will be used only for this testing and then discarded. Your names or any other identifiable information will not be shared or included in any report. For the analysis of study results, anonymous identification numbers will be used. Information and materials relating to the study must be retained for several years after its completion. Following this period, it will be destroyed in accordance with national and MSF regulations.

Patient responsibilities

By agreeing for your child to participate in this study, you confirm that you are the parent or legal guardian of the child. You also agree to providing information relating to your child's health requested by the nurse.

Study team's contact information

You may have questions about this diagnostic study. You are free to ask our nurse any questions you may have now. If you have questions later, you can call or write to Valyoat Gafurova MSF, Bukhoro Street 5-3, Dushanbe, 934440503: call Monday to Friday from 8:30 a.m. to 5 p.m.

Detection of Mycobacterium tuberculosis complex using Xpert MTB/RIF Ultra in the stool of paediatric patients

Ministry of Health and Social Protection and Médecins sans Frontières – Operational Center Amsterdam (MSF-OCA)

Participant informed consent

Patient identification number: _____

I _____ *(insert name of household head, father, mother, caretaker of child/ren here)* have been informed and have fully understood the purpose of this study and the samples that are being requested from the child in my care.

I understand the information and give permission for sputum and stool samples to be obtained from the child(ren) below for the purposes of testing for tuberculosis:

Child 1 *(insert name)* _____

Child 2 *(insert name)* _____

I understand these samples will only be used for the purposes explained to me.

I understand that I have the right to refuse without having to give a reason. I give voluntary consent for the participation in this study and understand that I am free to withdraw the child from the study at any time and face no penalty.

I have understood the information sheet and my questions have been answered to my satisfaction.

Date: ¦_D_¦ ¦_D_¦ / ¦_M_¦ ¦_M_¦ /¦_Y_¦ ¦_Y_¦

Caregiver's signature/fingerprint | Impartial witness signature (as applicable)

Caregiver's name | Impartial witness name (as applicable)

Testing team member name and signature:

_____

12.3 Annex 3. Assent form for children

Detection of Mycobacterium tuberculosis complex using Xpert MTB/RIF Ultra in the stool of paediatric patients

Ministry of Health and Social Protection and Médecins sans Frontières – Operational Center Amsterdam (MSF-OCA)

Child assent form

After speaking to you and your family, your doctors have decided to send you to have a test for tuberculosis. Normally, tests for tuberculosis are done in the laboratory on sputum that people might cough up when they are sick. We know that coughing up sputum can be difficult for younger children, so we would like to check whether the test for tuberculosis can be done on poo, since it is easier for children and their family to safely collect at home.

If you agree to help us learn whether this test can be done on poo, the nurse in the clinic will still help you collect a sputum sample as your doctor has requested.

We will give your parent or guardian some information about how to collect poo and ask them to bring it to the laboratory within one or two days of collecting the sputum in the clinic. You can do it in the clinic or at home. They will also be given information about how to contact us if you have any new questions.

We will share the results of tests with your doctor so that he or she can decide what is the best treatment for you. We will also call your parent or guardian after 6 weeks to see whether you required treatment and whether you are feeling better.

You can tell us at any time if you and your parent or guardian chose not to be involved in testing poo. The doctors and nurses will not be angry and will still look after you in the best possible way.

After some time, we hope that enough children and families will agree to have their poo tested so that we will know whether we should recommend this type of testing to others. We will mix together the test results from all of the children who agreed to help us and share them with other doctors and nurses in Tajikistan and in other countries. We will also explain the results to you and your family if you like. We hope that these results might make it easier for other children to be tested for TB more easily in the future.

If you think you would like to help us test your poo along with your sputum, would you be able to write your name below? Or tell the nurse or your parent when you visit the clinic?

Your name: _____

Date: _____

12.4 Annex 4. Information for parent or guardian on stool sample collection

1. *Why does my child need a stool sample tested?*

Your child has been referred to us because your child has shown signs and symptoms of tuberculosis. Specific tests need to be conducted in order to confirm or rule out this diagnosis. As you have decided to participate in a study conducted by MSF in collaboration with MoHS, a stool sample needs to be collected from your child to be tested for tuberculosis.

2. *What should I do?*

You will receive one or more special containers. Do not bring stool in any other container. Keep the containers out of children's reach.

Preparing to collect the stool

- For children in diapers, get 6-spoonful from the diaper, using the spoon from the stool container.
- For older children, put plastic wrap, newspaper, or a special collection container under the toilet seat or latrine to catch the stool. Place the plastic container or potty in the toilet bowl. Alternatively place clean newspaper or plastic wrap across the toilet seat opening.

Collecting the stool sample:

1. Allow the child to the stool into the potty, plastic container, or onto the newspaper or plastic wrap. Make sure it does not touch the inside of the toilet.
2. Take the caps off of the containers. Transfer 6 small spoonfuls of the stool into the blue specimen container using the spoon built into the lid of the specimen container. Never take the sample out of the water in the toilet bowl.
    - Do not overfill the specimen container. A walnut-sized amount, or a third of the container, is enough for testing.
    - Put on the specimen container lid and screw on tightly. If the outside of the container has got dirty, clean the outside with soap and warm water.

- Wash your hands thoroughly with soap and warm running water and dry.
- Dispose of the stool left in the potty, plastic container, newspaper, or plastic wrap into the toilet.
- Wash your hands thoroughly with soap and warm running water and dry.

3. Store properly and bring to the clinic as soon as possible:
    - Fresh samples: Keep in the refrigerator 4^0C and bring to Laboratory within 12 hours.
    - If no fridge, collect the sample in the morning on the day you are visiting the clinic. Store in a cool dry place and bring to Lab within 2hrs.

Questions?

This sheet is not specific to your child but provides general information. If you have any questions, please ask the referring nurse or call the clinic to: Valyoat Gafurova MSF, Bukhoro Street 5-3, Dushanbe, 934440503: call Monday to Friday from 8:30 a.m. to 5 p.m .

12.5 Annex 5. Laboratory standard operating procedure for handling stool specimens

Xpert MTB/RIF Ultra on Stool

Objective

Detection of *M. tuberculosis* complex DNA and RIF resistance screening in stool from paediatric suspects using Xpert MTB/RIF Ultra

Principle

The Xpert MTB/RIF ULTRA test for use with the Cepheid GeneXpert® System is a semi-quantitative nested real-time PCR in-vitro diagnostic test for:

1. The detection of the DNA of *Mycobacterium tuberculosis* complex in sputum samples or concentrated sediments prepared from induced or expectorated sputa that are either acid-fast bacilli (AFB) smear positive or negative
2. The detection of rifampicin resistance associated mutations of the rpoB gene in samples from patients at risk for rifampicin resistance. This Standard Operating Procedure is part of the study protocol submitted by Dushanbe Children Hospital.

Procedure originally published by Banada et al. (2016). A Novel Sample Processing Method for Rapid Detection of Tuberculosis in the Stool of Pediatric Patients Using the Xpert MTB/RIF Assay. Plos One, 11(3). doi:10.1371/journal.pone.0151980Plos one March 2016 (1)

Equipment

1. Biosafety cabinet class II
2. Laboratory Scale (ELAESCAE4)
3. Vortex mixer (ELAEVORE1)
4. GeneXpert System, 4-module plus a compute (ELAEMBIE4)

Material and Reagents

1. Gloves, disposable, not sterile.
2. Solid waste container
3. Wash bottle with disinfectant (prepared daily).
4. Absorbent paper
5. Sample container for stool, plast., 60ml, non-sterile, stools + spoon (STSSCONT6S-)
6. 50 ml conical tube sterile (ELABTUCE5SPP)
7. Glass beads 3 mm diameter (ELABBEGL003)
8. Transfer pipette, graduated, plastic, sterile, s.u (ELABPIPT1S-)
9. Syringe, 20 ml for stool process, s.u., sterile (SINSSYDL20)

10. Syringe 60 ml, s.u., for stool reagent preparation(SINSSYDL60)
11. Filter for syringe (stool reagent preparation), 0.20µm (ELABFILS20)
12. (mb GeneXpert) PYREX GLASS FIBER WOOL, 8µm pore (ELAEMBIC120)
13. Laboratory timer (ELABTIME1E)
14. Xpert MTB/Rif Ultra Cartridge (ELAEMBIT111) + Sample reagent (SR)
15. (mb GeneXpert) BUFFER AL, for DNA purification (ELAEMBIC118)
16. (mb GeneXpert) POLYVINYLPYRROLIDONE, powder 100g (ELAEMBIC119)
17. 50 ml conical rack (ELABTUCE5R)
18. Fine-point forceps (CWATVECTIFS)

Reagents

Stool Processing Buffer (SPB) - **prepare daily or weekly**

1. Weight 3 g of Polyvinylpyrrolidone in a 50-ml conical tube sterile
2. Add AL buffer up to 30 ml mark of the conical tube
3. Mix vigorously until the PVP is completely dissolved
4. *Note: PVP is difficult to dissolve and a lot of foam will form, help dissolving by placing the conical tube in a warm bath (~50^{o}C). Wait for the foam to settle before passing through the filter*
5. Sterilized the solution by passing it through a 0.20 um filter attached to 60-ml syringe into a new 50-ml conical tube. Label SPB with date of preparation
6. Cover the tube with foil and store at room temperature (the shelf life it is unknown it is better to prepare 50-ml each time and used within a week or two)

Preparation of the syringes with glass wool

Syringes fitted with glass wool - **prepare daily**

1. Use a clean room to prepare de syringes.
2. Make sure the working area is disinfected and clean.
3. Unpack a 20-ml syringe and remove the plunger, do not throw the packing material.
4. Take the glass wool using sterile fine-point forceps and place the wool inside the barrel of the syringe; put enough wool, loosely up to the 10-ml. mark.
5. *From the wool thread, cut the width of a finger*
6. Save the plunger and barrel in the syringe packing.
7. Prepare enough syringes to run the samples on a daily basis.

Biological samples

Fresh stool sample and/or fresh frozen stool sample.

Procedure (see figure below):

Fresh stool sample

1. Label the 50-ml conical tubes with the patient ID (2 tubes per stool sample). Label the tubes with the Lab ID (must be the same used for sputum sample) followed by “PROCESSING” for the first tube and Lab ID and “PROCESSED” for the second tube
2. Weight 0.6 g (+/- 0.2 g) of stool (do one sample at the time and clean the area in between)

(a) *Note: 4 scoops of the stool sample using the container spoon will give you approximately 0.8 g*

b. Work inside the biosafety cabinet.
c. Make sure the scale is balanced and calibrated (keep it inside the BSC).
d. Weight an empty stool container and tare.
e. Weight the stool container with the sample.
f. If the weight of the sample in the container is within the range:
    i. Add 2 ml of Xpert MTB/Rif Ultra Sample Reagent (SR) using a sterile transfer pipette
    ii. Mix thoroughly using the transfer pipette
    iii. Transfer with the same pipette to the 50-ml sterile conical tube labeled "PROCESSING".
g. If sample contains more than 0.8 g of stool:
    i. Weight an empty, sterile, pre-labeled (with Lab ID + "PROCESSING") 50-ml conical tube and tare
    ii. Take a small amount of stool specimen with a disposable knife (formed/hard stools) or transfer pipette with the tip pre-cut (watery stools) and place it in the bottom of the conical tube
    iii. Close and weight, wait until the scale stabilizes.
    iv. Repeat the process to add or remove stool until the scale reads 0.6 g (+/- 0.2 g).
    v. Add 2 ml of Xpert MTB/Rif Ultra SR using a sterile transfer pipette
    vi. Mix thoroughly using the transfer pipette.

3. Add 2 ml of SPB
4. Add approximately 10 glass beads and close tube
5. Do this with all stool samples to be processed in the batch (max 4)
6. Vortex at maximum speed until stool appears dissolved (~ 1 min)
7. Incubate for 30 min a room temperature.
8. Transfer the whole content of the 50-ml conical tube (except the glass beads) inside the syringe fitted with the glass wool. Pass the content into a new, sterile, pre-labeled (Lab ID + "PROCESSED") 50-ml conical tube.
9. Add 2 ml of this filtrate into a Xpert MTB/RIF Ultra cartridge
10. Process the cartridge as for sputum samples

Frozen stool sample

1. Remove sample (stool) from refrigeration
2. Place sample on bench work and let it thaw for 3 hours.
3. Once sample has thawed
4. Follow the procedure as above for fresh stool samples.

References

Banada et al. (2016). A Novel Sample Processing Method for Rapid Detection of Tuberculosis in the Stool of Pediatric Patients Using the Xpert MTB/RIF Assay. Plos One, 11(3).

Weigh stool (0.4 – 0.8 g)

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Add 2 ml of Stool processing buffer (SPB)+ 2 ml of SR (Xpert MTB/RIF)+~10 glass beads and snap vortex

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Incubate for **30 min at room temperature**

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Pass the sample through the glass wool syringe filter in to a separate container →

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Add 2 ml of this filtrate into Xpert MTB/RIF assay cartridge ←