Improving diagnosis of childhood tuberculosis in a high TB-HIV prevalent setting

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Improving diagnosis of childhood Tuberculosis in a high TB-HIV prevalent setting

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Sous la direction de Dr. Maryline Bonnet Directeur de thèse

Devant le jury composé de

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Dr. Jean-Francois Etard, President of the Jury,

I have made all the corrections suggested by the jury. In summary the following issues were raised by the jury and have been addressed:

- To include incipient infection and subclinical TB disease spectrum: Literature on this new disease spectrum was added as shown on page 18 and 19 of the manuscript.
- To include the IGRA test among the new promising methods: A section has been added to show sensitivity of the IGRA as shown on page 41.
- To include the oral swabs among promising sample collection methods: A section has been added to show sensitivity of the oral swabs in a South African study as shown on page 47.
- To revise the text and edit the gramma accordingly. The entire manuscript was revised based on comments from the Jury.
- To remove duplicate blank pages. These errors were corrected and blanks removed.
- To re-scan the articles in the research section. Better scanned copies have been added in the research section of the manuscript.

DEDICATION

THIS THESIS IS DEDICATED TO ALL VULNERABLE CHILDREN FROM LOW RESOURCE COUNTRIES IN DIRE NEED OF A RAPID, AFFORDABLE AND EASY DIAGNOSTIC TEST FOR TUBERCULOSIS.

QUOTE

"If I saw further than others, it is because I was standing on the shoulders of giants''

Sir Isaac Newton (1642-1727)

Acronyms

ART	Anti-retroviral treatment
AFB	Acid fast bacilli
BCG	Bacillus Calmette-Guerin
CPC	Cetyl-pyridinium chloride
CXR	Chest X-ray
DNA	Deoxyribonucleic Acid
DR-TB	Drug resistant tuberculosis
EPTB	Extra Pulmonary Tuberculosis
FIND	Foundation for Innovative New Diagnostics
FM	Fluorescent microcopy
GHI	Global hunger index
HAART	Highly active antiretroviral therapy
HBC	High burden countries
HIV	Human Immunodeficiency Virus
IGRA	Interferon gamma release assays
IPT	Isoniazid preventive therapy
LAM	Lipoarabinomannan
LAMP	Loop-mediated isothermal amplification
LED	Light-emitting diode
LJ	Lowenstein Jensen
LPA	Line probe assay
LTBI	Latent tuberculosis infection

MDR TB	Multi-drug resistant tuberculosis
MGIT	Mycobacteria growth indicator tube
MTB	Mycobacterium tuberculosis
NAA	Nucleic acid amplification
NALC	N-acetyl cysteine
NAOH	Sodium hydroxide
NPA	Nasopharyngeal aspirate
OADC	Oleic albumin dextrose catalase
PANTA	Polymyxin, amphotericin B, nalidixic acid, trimethoprim and azlocillin
PCR	Polymerized chain reaction
POC	Point of care
PTB	Pulmonary tuberculosis
SAM	Severe acute malnutrition
SDG	Sustainable development goals
TB	Tuberculosis
TST	Tuberculin skin test
WHO	World Health Organization
ZN	Ziehl- Neelsen

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CHAPTER 1

INTRODUCTION

1.1 Background

1.1.1 Mycobacteria and its general characteristics

Tuberculosis (TB) is an infectious disease that is caused by *Mycobacterium tuberculosis* complex (MTBc) belonging to *Mycobacterium*, a very unique genera comprising of 71 recognized species. Members of this group include pathogenic Mycobacteria such as MTB complex that comprises of *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis,* and *Mycobacterium microti. Mycobacterium leprae*, *Mycobacterium ulcerans* are distinct from other members of *Mycobacterium* due to the degree of pathogenicity in humans.

Structurally, mycobacteria are rod-shaped, slightly curved or straight organisms, measuring 0.2x0.6mm by 1.0x10mm in size (1). They are non-motile, with a unique cell wall that has an extremely high lipid content. This unique cell wall feature makes mycobacterial cells characteristically resistant to staining with commonly used basic aniline dyes at room temperature and has been exploited in the microscopy techniques. The organisms take up aniline dyes with increased staining time or application of heat and resist decolourisation with a strong acid and alcohol mixture, a property referred to as acid fastness or alcohol acid fastness (2). Other than diagnosis, the high lipid content is defensive as it assists the MTBc and non-tuberculous mycobacteria (NTM) to resist many antibiotics, acids and alkaline compounds. This partly explains the long TB treatment duration and slow growth of MTBc (up to 8 weeks) in highly enriched laboratory culture media.

Not all members of the Mycobacterium genus however are primary pathogens to humans. None or less pathogenic Mycobacteria are referred to as NTM. These NTM may also be referred to as: environmental mycobacteria, atypical mycobacteria, or mycobacteria other than tuberculosis (3). Most of these are generally free-living microorganisms with some species, such as *M. smegmatis* occurring saprophytically while others including *M. avium* are opportunistic pathogens, causing disease only in immune-compromised individuals. Recently, with more advanced diagnostic methods, many of these NTM including *M. kansasii*, *M. abscessus*, *M. fortuitum*, and *M. chelonae* are increasingly isolated from patients with compromised immunity (4,5) where they are

occasionally associated with pulmonary disease, lymphadenitis, cutaneous disease, and disseminated disease (3).

Even though these NTM may occasionally cause infection, most of the cases of mycobacterial diseases in disease endemic countries is always due to MTBc. Despite a clear understanding of the structure, mechanisms and characteristics of the organism, MTBc continues to pose challenge in diagnosis as the disease increasingly cause havoc to humanity and to some animals. There are tools that are currently in use with good accuracy and reliability that have helped in identifying TB patients to initiate timely treatment. However, these tools have failed to address the problem of poor reporting as World Health Organization (WHO) reports continue to show low notifications rates over the years.

1.1.2 Tuberculosis infection and disease

Tuberculosis (TB) causes unbearable suffering in the whole world and is one of the top 10 causes of death but also the leading killer disease from a single infectious agent (6). This is because annually, millions of people are infected with the disease leading to a high proportion of deaths. Originally called "consumption" (7) due to weight loss of the individual, this ancient disease, continues to cause untold public health outcry, along with social economic challenges as a result of significant depletion of global health budget and loss of productivity. TB is known to have major impact on the economically active age group (8).

MTBc microorganisms have high affinity for oxygen and therefore flourish within the respiratory system, even if they may also infect other body sites. Due to this unique habitat, MTBc bacilli are easily spread through air as a result of coughing, spitting, even talking where droplet nuclei contained in aerosol are generated from the lungs (9). Infection occurs when a person gets exposed to aerosols containing droplet nuclei (1– 5 microns in diameter) of MTBc that can reach the lung alveoli. Given this model of natural history, in every high TB prevalent setting, there are three potential risk points that can be identified: the risk of acquiring infection, the risk of developing progressive primary disease once infected, and the risk of reactivation of latent infection. Historical evidence has shown that the risk of acquiring infection is associated with the concentration of

organisms in the sputum, frequency of cough in the index person, duration and proximity of exposure to an infectious case, and on the infectiousness of the source (10–13). Patients with active pulmonary TB often have bacilli present on smear and are more likely to transmit infection to a naive contact than smear negative counterparts. Notably, nodular lesions are known to have approximately 100-10,000 organisms, unlike cavitary lesions which have 10 million to 1 billion bacilli. Thus the risk of acquiring infection from a cavitary lesion is much higher and easily coughed through sputum indicating that contacts of such persons have an increased chance of acquiring infection. The other risk factor for acquisition of infection is related to the environment where the concentration of bacilli depends on the ventilation system of the surroundings. In that respect, communities with likelihood of overcrowding such as prison settings, slums with poor housing facilities and inadequate ventilation are hotspots that may predispose children to exposure and development of TB infection.

Upon inhalation, the bacilli are deposited (usually in the midlung zone) into the distal respiratory bronchiole or alveoli, which are subpleural in location. Subsequently, the alveolar macrophages phagocytose the inhaled bacilli. However, these naïve macrophages are unable to kill the mycobacteria, and the bacilli will slowly multiply in terminal alveoli of the lungs and corresponding lymph node representing the primary complex (Figure 1).
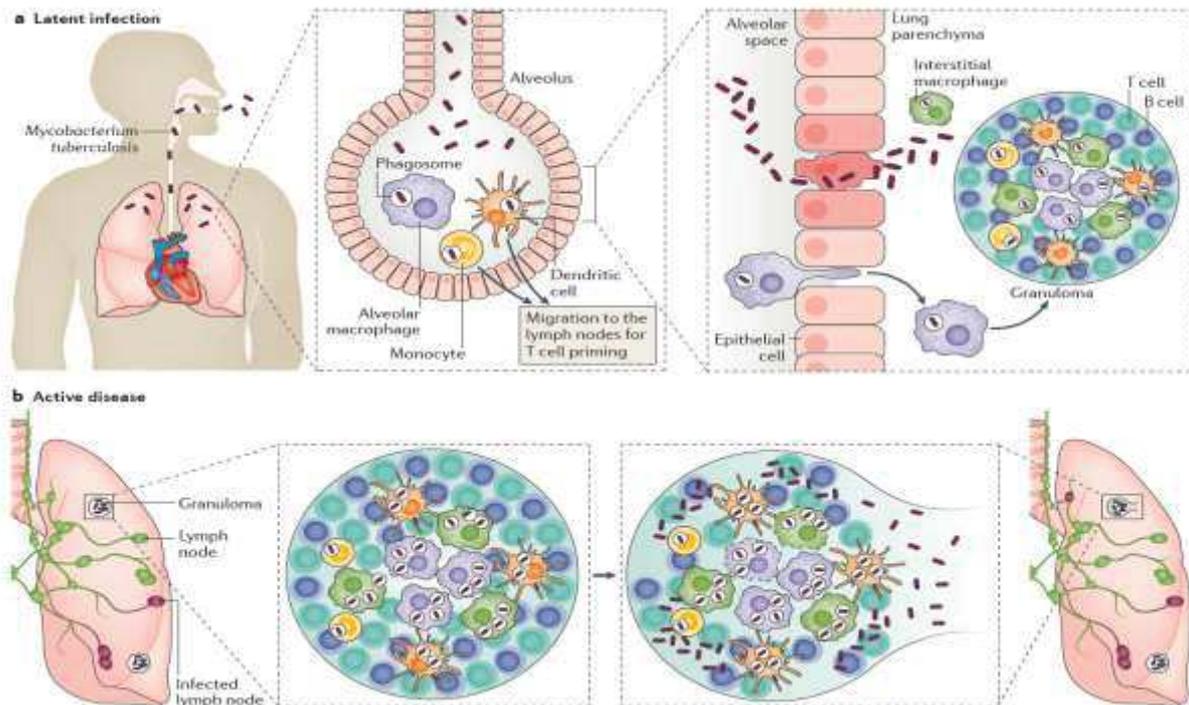
Figure 1: Tuberculosis manifestation in the human system. Pai et al., 2016(14)

Within 1-2months, the complex will encapsulate and contain caseous necrosis due to cell-mediated immunity. In majority of cases, the infected person will be asymptomatic, bacilli remaining dormant in macrophages and infection could be diagnosed by either tuberculin skin test or interferon gamma release assay. Then the individual is considered to have latent or persistent infection. In some cases, the enlargement of the caseous area may lead to pneumonia, atelectasis, and air trapping. This is more likely to occur in young children causing fever, cough, malaise, and weight loss.

With new understanding of TB pathogenesis, the traditional understanding of the cycle has changed over time. Indeed, the risk of transition from latent TB to active disease and the speed of progression to clinically detectable disease depends largely on the immune competency of the host. Emerging evidence is pointing at several pathophysiological mechanisms involved before TB disease becomes evident. With increased sophistication of diagnostic tools, it has become clearer that some asymptomatic persons may reveal radiological and or microbiological evidence of

disease much before disease manifestation (15). Thus terms such as ''incipient'' and ''subclinical'' TB have become increasingly used to describe asymptomatic persons with radiologic and/or microbiologic evidence of TB, who could be having early stages of TB that may progress into disease manifestation. As shown in figure 2, incipient and subclinical TB are the intermediate stages in the transition from latent TB to active TB and differ in duration from person to person. Incipient TB is characterized by an infection with viable MTBc bacteria that is likely to progress to active disease in the absence of further intervention but has not yet induced clinical symptoms, radiographic abnormalities, or microbiologic evidence consistent with active TB disease(16).

Sub clinical TB on the other hand is disease due to viable MTBc bacteria that does not cause clinical TB-related symptoms but causes other abnormalities that can be detected using existing radiologic or microbiologic assays(16).

The difference between these latent TB and the two; incipient or subclinical TB is that, with latent TB, disease progression is highly unlikely in the near future unless the body immunity weakens. However, with incipient and subclinical TB, evidence from IPT studies show majority of patients with subclinical TB developed TB manifestation within 3 months of screening signifying the relevance of early stage of this diagnosis (17).

Active TB on the other hand is disease due to viable MTBc that causes clinical symptoms with radiographic abnormalities or microbiologic evidence consistent with active TB disease (18). Thus active TB is characterized by symptomatic manifestation of disease with evidence of radiological or microbiological of viable MTBc.

*Rising TB burden implies an increase in abundance of TB and pathogen biomarkers, compartment-specific changes in immunological responses, and a decrease in the probability of disease resolution in the absence of treatment.
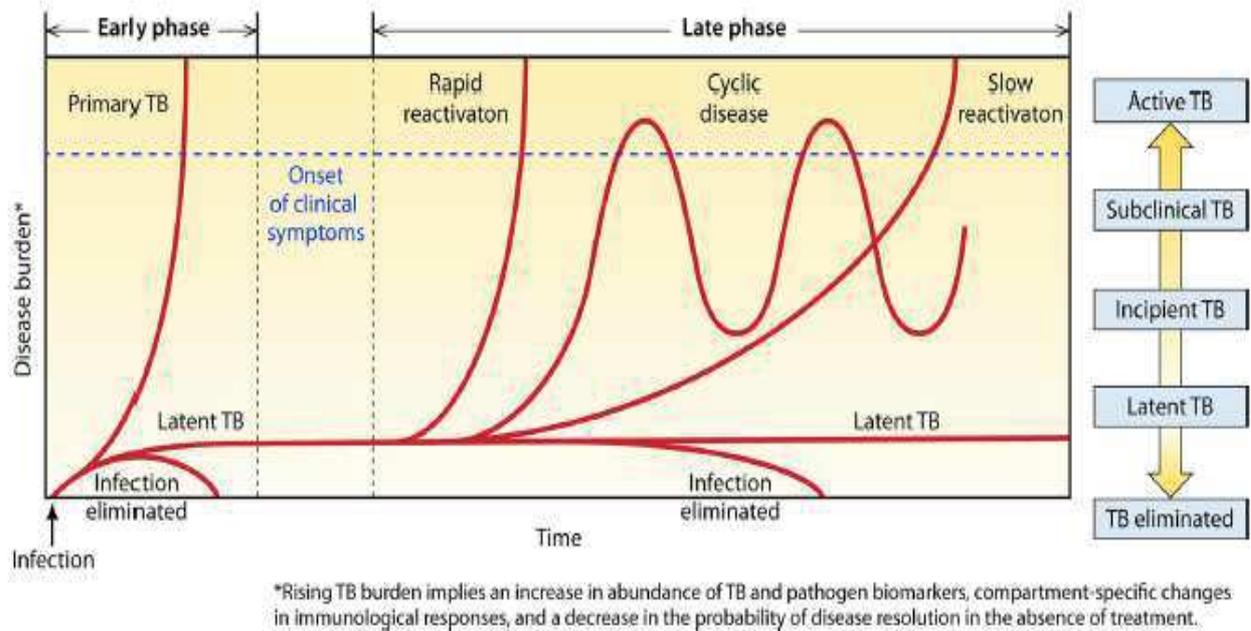
Figure 2: Pathways of tuberculosis disease progression. After initial exposure, *M. tuberculosis* may be eliminated by the host immune response, persist as a latent infection, or progress to primary active disease. Following the establishment of latent infection, disease may persist in a latent form, naturally progress in a slow or rapid fashion to active tuberculosis, or cycle through incipient and subclinical states before developing into symptomatic disease or eventual disease resolution. Although not all possibilities for regression of disease burden are depicted, spontaneous recovery may occur in any of these clinical trajectories(16).

Once infected, the risk of developing disease is heterogeneous because intrinsic characteristics of the host modify the risk of disease during the long and variable latency period that in most cases depend on the host immunity. The initial host immune response to infection may either contain the initial infection or not. If the initial immune response fails to contain the infection, the individual develops progressive primary disease. A lower proportion (10%) of those infected will progress to active disease in their lifetime, unless the host immune system is suppressed.

A few adults that develop active disease often get it many years after the initial infection due to reactivation of latent foci of infection. Some of the risk factors that predispose TB disease include Human Immunodeficiency Virus (HIV), undernutrition, severe acute malnutrition, diabetes,

smoking, alcohol consumption, bacterial load, young age and elderly condition. In young children, there is 40-50% risk of disease progression in the first 2 years of life (19).

Majority of people with TB disease have a subacute clinical presentation of nonspecific general signs including weight loss, night sweats, loss of appetite, fever and fatigue combined with signs related to the site of the TB disease, which in the lungs is 80% of cases. Therefore, chronic or non-remitting cough is one of the most common appealing sign of TB.

In children, primary intrathoracic disease is the most common manifestation mostly characterized by mediastinal adenopathy; seen in more than 50% of children with asymptomatic TB infection as a transient phenomenon (20). The cavitating lung disease that is common in adults is mostly seen in older children from early adolescent years onwards(21). Reactivation disease usually presents during adolescence and is most common in areas endemic for TB and in HIV infected patients (19). Unlike older children, infants develop disease much faster after infection indicating that most confirmed or clinically diagnosed cases can be associated with recent or ongoing transmission. Clinical presentation may be more acute, mimicking those of acute severe, recurrent or persistent pneumonia. In very young children or immunocompromised children, non-pulmonary TB can be more frequent, in particular lymph node TB, meningitis or disseminated TB.

Luckily, TB is curable and can be treated using 6 months of treatment combining antibiotic either bactericidal or bacteriostatic. Treatment for adults or children requires an intensive phase of 2 months of daily ethambutol (E), isoniazid (H), rifampicin (R), and pyrazinamide (P) followed by 4 months of daily isoniazid and rifampicin (2EHRZ/4HR) using fixed dose combination (FDC) (18). Children living in low HIV prevalence settings, low prevalence of isoniazid resistance, and who are HIV-negative can be treated with HRZ for 2 months followed by HR regimen for 4 months(22). In 2010, based on pharmacokinetic data WHO has revised the anti-tuberculosis drug dosage for children increasing the dose per body weight of R, H and Z and since 2017, child-friendly FDCs (R75/H50/Z150 and R75/H50) are available for treatment of children weighing less than 25 kg(23). These new water-dispersible tablets with a pleasant taste, offer the opportunity to

simplify and improve treatment for children around the world and are therefore likely to enhance adherence and completion of treatment, as well as to prevent the development of drug resistance.

The long duration of treatment remains a challenge to ensure good treatment adherence and may result, in some cases, emergence of drug resistance and multi drug resistant TB (MDR-TB); defined by resistance to at least rifampicin and isoniazid, two of the most effective drugs used in first line TB treatment. In the 2018 WHO report, MDR-TB prevalence was estimated to be 3.5% among new TB cases and 18% in previously treated patients(6). This complicates the management of TB due to longer duration of treatment and drugs' toxicity (24).

As part of treatment, immunization against tuberculosis still relies on the Bacillus Calmette-Guerin (BCG) vaccine that was introduced to prevent the disease in 1908 (25). The BCG vaccine protects against TB meningitis and other severe forms of TB in children below 2 years but has a low overall TB protection and is recommended at birth in all high TB burden countries (24,25). Trials are currently evaluating new vaccines and boosted BCG vaccines (26). Preventive therapy of exposed people identified with latent TB infection, defined as a state of persistent immune response to stimulation by MTB antigens with no evidence of clinically active disease (27), is also used to reduce the risk of developing TB disease. Preventive treatment includes 6 months regimen of isoniazid or daily 3 months regimen of isoniazid-rifampicin or weekly rifampentine-isoniazid for 3 months(28). Primary prophylaxis is recommended for people at high risk of activating TB if infected such as people living with HIV from high TB burden countries and contacts (28).

1.2 Epidemiology

1.2.1   Global tuberculosis burden

TB affects all countries and all age groups as a result of continuous transmission. In the 2018 report, WHO estimated that there were 10 million new cases of TB globally, and 1.3 million deaths among non HIV infected and 300,000 among HIV infected persons (6). It is reported that 90% of all cases were adults (aged ≥15 years), among whom 64% were male and 9% HIV infected (72%

in Africa). The 1.3 million deaths translates to five thousand people dying from TB every day. These estimates raise more concern about the danger associated with MTB, and partly explains why TB is a leading cause of death from a curable infectious disease.

Remarkably, the disease incidence is geographically distributed but most cases occur in eight particular hotspot countries that account for two thirds of the burden and these include: India (27%), China (9%), Indonesia (8%), the Philippines (6%), Pakistan (5%), Nigeria (4%), Bangladesh (4%) and South Africa (3%)(6). In these hotspots and other high burden countries, up to 150-400 incidences per 100 000 population occurred in 2017. In most cases, poor communities experience more chances of developing TB especially as a result of continuous transmission majorly due to socioeconomic and behavioral factors. Majority of poor settings occur in Sub-Saharan Africa where limited resource concerns result in increased social mixing due to overcrowding, creating a recipe for transmission of TB at a much higher rate (29). In addition, the HIV pandemic, and other conditions that alter the immune response, have increased the risk of disease progression. Diabetes, alcohol, malnutrition, tobacco smoke, and indoor air pollution are other factors that may accelerate progression to TB disease. Figure 2 shows the incident cases across the globe in 2017.
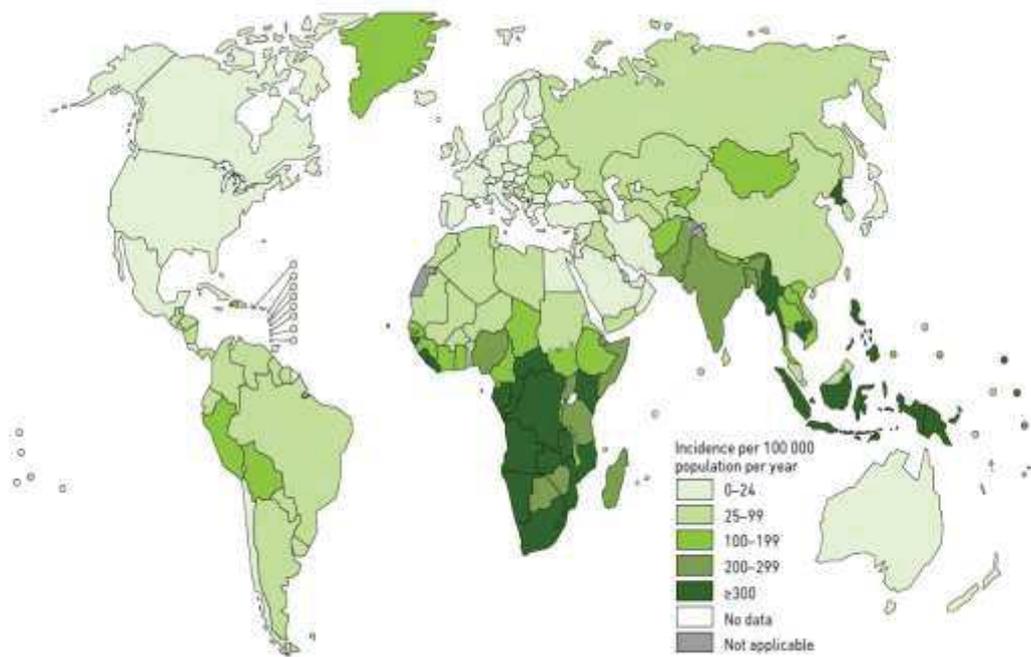
Figure 3: Estimated tuberculosis incidence rate, 2017. Source: WHO TB Report, 2018

Despite the high number of cases estimated each year, only 6.4 million (64%) were notified to national programs among new and retreatment cases globally (6). This leaves 36% of TB patients unaccounted for in the diagnosis or treatment cascade, who continue to transmit TB unnoticed leading thereby further escalating the TB problem. Multiple systemic reasons can explain this repeated gap in detection or notification including, lack of access to diagnosis because of; unavailability of adequate health infrastructure, lack of equipment, limited supplies, poor health care seeking behavior, poor quality of specimen and absence of personnel at health facilities among other things and these play a key role in the outcome.

Although Uganda is no longer among high burden countries, it is currently one of the 30 high TB-HIV burden countries (6). The actual prevalence of TB in the country is doubtful. The population TB prevalence survey, conducted in 2015, reported an estimated incidence of TB of 234/100,000 population for all TB cases and prevalence of 253/100,000 population (30,31). This is much higher than the estimated incidence of 201 per 100 000 people reported by WHO based on notified cases

by the national TB control program in 2017 (32). TB kills both HIV and non HIV people in Uganda with case fatality rate estimates of 26 (95CI:14-42) per 100 000 people as in 2017 (32).

In addition, 40% of TB notified cases occur in HIV infected patients (32). Among the HIV infected alone, TB killed approximately 12/100,000 people.

Although TB treatment is free by government of Uganda through the Global Health Initiative, the first national TB costs survey reported high expenditures, that cause untold suffering during medical visits for diagnosis and treatment and buying nutritional supplements (33).

1.2.2   Pediatric tuberculosis burden

Children largely contribute to the global TB burden as the disease can manifest at any stage of growth and development. Each year, there is a consistent number that is reported to acquire the disease and majority occurs in low middle income settings. According to WHO estimates there is a consistently high annual incidence of close to 1 million cases among children below 15 years of age with about 230 000 deaths (6,34–36). These figures are alarming considering that this is equivalent to nearly 650 childhood deaths from TB every single day (6). Countries with high burden of TB are more likely to suffer worse incidences and subsequently poorer outcomes. Indeed, up to 75% of global pediatric TB burden has been reported to occur within the 22 high burden countries (37). In those settings, it is estimated that of all TB cases, paediatric TB accounts for up to 20% (38).

WHO report noted that higher incidences and relapses occurred mostly in East, Central and southern Africa, Asia and South America (Figure 3).

* 2016 data were used for 23 countries.
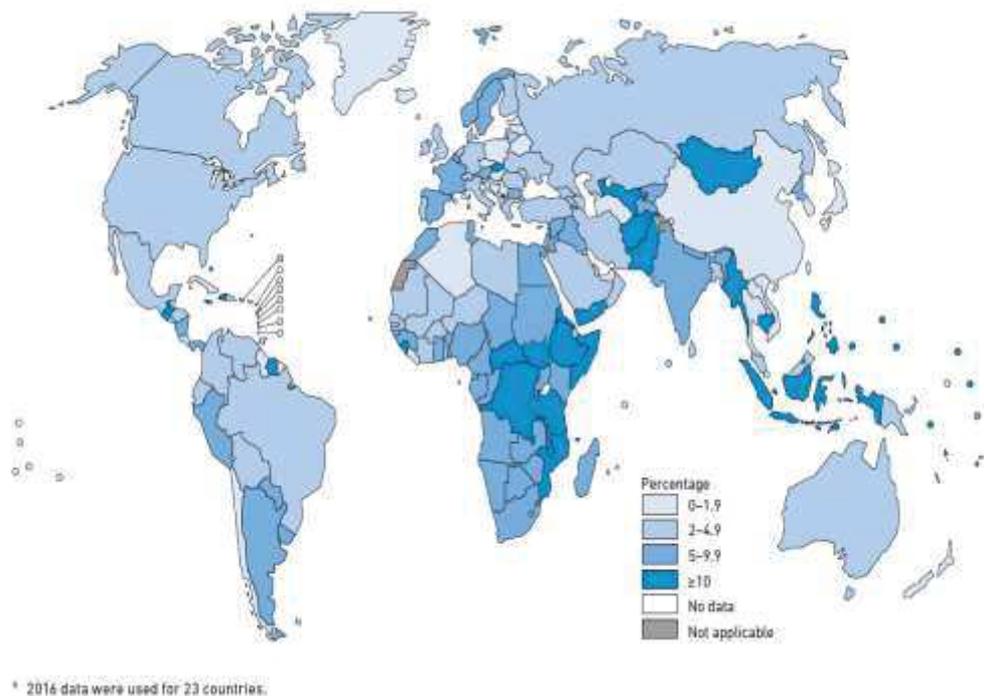
Figure 4: Percentage of new and relapse TB case that were children, 2017. Source: WHO TB report, 2018

At the same time, these figures are highly subjective as only less than 50% of pediatric TB cases are notified annually as a result of inadequate investigative options or poor notification. This raises a major concern since these children will miss timely treatment or arrive late with several comorbidities that will likely cause high mortality or related poor outcomes. Mathematical models predict that 96% of children who died of TB within a year did not receive treatment compared to their counterparts that received treatment with a much lower risk of 1% (39). This could explain the high childhood TB mortality risk projection of more than 80% that has been reported through mathematical models (40).

For children, in particular young children, MTB exposure occurs in the household, which support the concept of systematic child contact tracing of any adult diagnosed with TB, especially with bacteriologically confirmed TB and the prioritization of young children and HIV infected children for preventive therapy in high burden and poor resource countries(28). Both contact tracing and

preventive regimen have been poorly implemented in many countries. A number of challenges have been advanced for low implementation and some strategies are proposed to improve its coverage (38, 39). This includes the WHO recommendation to use a simple symptom based screening to exclude active TB in child contacts and the initiation of preventive therapy for asymptomatic children at high risk of developing TB disease without further confirmation of TB infection (28).

In addition to the immaturity of the young children immune system, non-TB infections, such as measles, varicella, and pertussis, may activate quiescent TB in children. The most at risk age group in TB-endemic countries has been reported as infants and younger children between 1 and 4 years where pulmonary TB is the most prevalent and with highest risk of developing severe forms or disseminated disease that may present as TB meningitis or miliary TB associated with a high morbidity and mortality (21).

In children, although pulmonary TB is the most prevalent, the disease is rarely bacteriologically confirmed due to difficulty in obtaining a proper specimen as children cannot easily cough out sputum. In a few instances, there can be haematogenous dissemination of the organism especially in infants and younger children. This may spread throughout the body resulting in acute disseminated (i.e miliary) TB, affecting any tissues including bones, brain, meninges and abdomen. This is termed as extrapulmonary TB (EPTB) and occurs in approximately 20–30% of all cases in children of which TB adenitis and TB pleural effusion are the most frequent forms. TB in children can get even more complicated if not well managed or if they acquire a resistant strain. Indeed MDR TB is an emerging challenge in the management of TB in children. The actual burden in children is not well known as laboratory diagnosis particularly drug susceptibility testing remains poor and most estimates available for only adults (8). However, mathematical models estimate that out of the 67 million children who could be infected with MTB, 2 million might be infected with MDR TB strain (43).

In Uganda, the actual burden of pediatric TB is not clearly understood. The main challenge, like in many other low resource settings, is lack of appropriate diagnostic methods. However, using clinical and laboratory diagnosis, the national TB survey reported a high prevalence of up to 36

cases per 100,000 population in children (30). The same survey reported the number of children below 5 years, from household contacts of bacteriologically-confirmed TB cases, receiving preventive treatment as 8.4% (7.7–9.2). Other supporting evidences have consistently shown low number of confirmed cases below the global estimates.

1.2.2.1 Pediatric tuberculosis and HIV infection

According to UNICEF report, there was a global estimate of 36.7 million people living with HIV in 2016 and among these, 2.1 million were children below 15 years of age (44). TB is the most common opportunistic infection in people living with HIV. HIV plays a huge role in the risk of acquiring TB disease progression because of depleted immunity, making them highly susceptible to opportunistic infections (45). Most of them develop TB as the first opportunistic manifestation of AIDS leading to poor outcomes.

In the WHO TB report, 10% of total deaths was among children living with TB and HIV(6). This is slightly lower than 17% that was reported in 2015 (40) indicating some slow progress in reverting the situation. There is further evidence showing that HIV infected persons are 20 to 30 times more likely to develop active TB than HIV uninfected counterparts (6). Thus children living with HIV infection face much higher risk of TB exposure, infection, progression to disease, and TB-related morbidity and mortality (22). It has been shown that children with HIV have up to a tenfold greater risk of dying from TB than children with TB alone (46).

With timely, antiretroviral therapy (ART) intervention, TB treatment outcomes are improved as a result of restoring immune function (47). A meta-analysis evaluation of impact of HIV, ART on TB risk in children, observed that ART was strongly protective against TB, but took 2 years to achieve full protection (48). It is therefore evident that HIV and TB form a big challenge to health burden especially in low resource settings where access to ART is limited by nonfunctional health systems including supply and structural concerns. The main challenge is that there is still poor coverage of ART as only half of eligible children are currently on treatment compared to 60% in adults(49).

Because of the dangers associated with TB-HIV synergy, WHO recommends regular TB screening of all HIV infected children in a TB-endemic setting, at each visit to a health facility or contact with a health worker (22). The main goal is to identify those patients who are likely to have TB disease, requiring anti-TB treatment, and those who should start IPT.

Systematic screening for TB and HIV among children with contact history is an important preventive measure including IPT.

In Uganda, according to a recent survey, the prevalence of HIV among children aged 0-14 was 0.5% corresponding to approximately 95,000 children living with HIV (50). The IPT guidelines recommend that screening of TB disease in children with HIV should be initially based on the presence of clinical symptoms followed by laboratory assessment. Among children living with HIV aged 12 months and above should be routinely given IPT after excluding active TB disease (51). HIV infected children below 12 months of age, with a history of household or close contact with a TB patient should also be given IPT after excluding active TB disease.

1.2.2.2 Pediatric tuberculosis and malnutrition

Malnutrition and TB form another important challenge in the paediatric TB management mainly leading to poor treatment outcomes. Malnutrition is determined through measurement scales defined by WHO. Unlike adults, young children are more prone to malnutrition because of high protein and energy needs coupled with susceptibility to infections (52). Malnutrition continues to ravel children across the globe with high numbers reported over the years. According to a joint UNICEF, WHO, and World Bank report, approximately 149 million (21.9%) children under 5 years were stunted, while 49.5 million (7.3%) were wasted, and 16.6 million (2.4%) severely wasted in 2018 (53). This is supported by more evidence gathered from the Sustainable Development Goals (SDG) report highlighting an increased number of undernourished people from 777 million in 2015 to 815 million in 2016 (54).

In most poor countries, malnutrition is a result of insufficient dietary consumption. Hunger is still a major concern, despite the SDG goal of ending hunger, with Global Hunger Index reporting 50

countries mostly in Africa and Asia, in that docket (55). Thus hunger and malnutrition pre-dispose children to infectious diseases leading to increased risk of under-five mortality.

It has been shown that nutrition is significantly poorer among children with active TB compared to healthy ones (56). At the same time, macronutrient and micronutrient deficiencies increase the risk of developing TB with poorer outcomes including higher mortality and delayed recovery compared to well-nourished one (56,57).

Severe wasting (weight for height Z-score (WHZ) < –3 standard deviation) or severe acute malnutrition (SAM) usually indicate recent and severe weight loss resulting from hunger and/or disease. The pathophysiology of TB and malnutrition maybe explained by a deficit in innate immunity that majorly contributes to progression to TB disease through reduced proliferation of T-cells and impaired cell-mediated immunity, phagocyte function, complement system, secretory immunoglobulin A antibody concentrations, and cytokine production which in turn leads to increased susceptibility to infections (58). A study reported great susceptibility to infections including TB leading to high prevalence of TB (22%) among children with severe malnutrition in Asian populations (59).

One quarter of all cases of wasting have been reported in Africa. In 2018, 14 million children were estimated to be wasted including 4.2 million severely wasted (53).

In line with these high malnutrition cases in Africa, studies have explored the link to TB disease progression in children with reports consistently showing poor treatment outcomes. In adults for example, it has been shown that moderate-to-severe malnutrition is associated with high risk of mortality within the first 4 weeks of TB treatment (60). Few studies conducted among children with malnutrition-TB and comorbidities including HIV infection in different countries of Africa also reveal high mortality among those started on TB treatment (61–63). Malnutrition and severe radiographic findings were associated with unfavorable outcomes most cases of death occurred within 18 months of initiating treatment (58).

Similarly, Uganda faces a challenge of malnutrition and TB among children. Despite abundancy of food in some parts of the country, malnutrition has continued to disturb the population with approximately 2.2 million (29%) under five children stunted (64). The cause of stunting has been

associated with growing under limited food provision and poor health care, despite 72.4% of households considered to be reasonable food secure (65). In addition, malnutrition is driven by lack of access to clean water and sanitation, high disease burden; especially childhood diarrhea and malaria, poor infant and young child feeding practices. Even areas in southwestern Uganda, which are considered as "food basket" due to high production of staple foods, have experienced some of the highest rates of stunting among children under 5 years in the country (64). In addition, there has been reports suggesting that Ugandan diet lacks diversity and fails to provide sufficient micronutrients (65). One of our studies reported a 9.86 prevalence of severe malnutrition in a rural population in southwestern Uganda associated with a TB burden of 13% within a regional hospital (66). Thus the impact of TB and malnutrition cannot be ignored, is likely bigger than estimated, and could easily increase and worsen the proportion of TB and its outcomes.

1.2.3 Tuberculosis management response

The SDG defined by the United Nations are the blueprint to achieve a better and more sustainable future for all. Goal 3 of SDG emphasizes good health and wellbeing by ensuring healthy lives and promoting well-being for all age groups. In line with this goal, the vision for global tuberculosis strategy is "*a world free of tuberculosis*", also coined "*zero deaths, disease and suffering due to tuberculosis*" with the ultimate goal of ending the global tuberculosis epidemic by 2035 (67). Indeed the SDG reports progress in reduction of TB but still calls for more action if we are to achieve these goals. For example, globally, there were 140 new cases of tuberculosis per 100,000 people in 2016 compared to 173 cases per 100,000 in 2000 (54).

In 2015, in order to achieve the Goal 3 of the SDG for tuberculosis, the WHO has proposed its new strategy, the End TB strategy that aims to end the global TB epidemic, with targets to reduce TB deaths by 95% and to cut new cases by 90% between 2015 and 2035, and to ensure that no family is burdened with catastrophic expenses due to TB (68). Pillar 1 of the End TB Strategy is "Integrated, patient-centered care and prevention" that has four components: i) early diagnosis of TB including universal drug susceptibility testing (DST), and systematic, screening of contacts

and high-risk groups; ii) treatment of all people with TB, including drug resistant TB, and patient support; iii) collaborative TB/HIV activities, and management of comorbidities; and preventive treatment of persons at high risk, and vaccination against TB (67). This has been further emphasized in the compendium released by WHO (69), further emphasizing the importance of pediatric TB on the global scale.

In response to this pillar, it is important to address early detection of contacts of persons with TB and primary prophylaxis of patients at risk of developing disease such as people living with HIV and contacts (69). Besides screening, diagnosis is key to confirm the disease with the use of new rapid molecular assay from sputum of patients with presumptive TB as a front line diagnostic test (70). With these kind of interventions, it will be possible to reach the milestone and the targets set by the end TB strategy. As rightly stated this will require working with communities, civil society and all partners, governments need to assume full responsibility for ensuring person-centered, modern, high-quality TB services and securing comprehensive care along with essential support for each person with TB, which also calls for collaboration within and beyond the health sector(10). This comprehensive package calls for strong partnerships and interventions to render universal health coverage. To further emphasize the urgency to eliminate TB, the theme of World TB Day 2019 '*It's time'* was carefully chosen as WHO launched a joint initiative dubbed "*Find, Treat, All"* campaign with the Global Fund and Stop TB Partnership, with the aim of accelerating the TB response and ensuring access to care, in line with WHO's overall drive towards Universal Health Coverage(71). With proper diagnosis and using different methods in all age groups, we can achieve these SDG milestones. The WHO end TB strategy responds to SDG by advocating for an aggressive pursuit of research and innovation to promote development and use of new tools for tuberculosis care and prevention (72).

Considering that TB is associated with poverty, the success in TB reduction may be undermined by the threat from catastrophic costs, defined as spending at least 20% of household income on TB care, which may affect the treatment outcomes. Using models, it has been shown that without extreme poverty, a 33% reduction in global TB incidence would be achieved by 2035 (6). This therefore further emphasizes the need to find fast diagnostics to reduce on waiting time for patients,

reduce costs and transmission pattern. The amount of resources used in diagnosis, treatment (medical and supportive) and time spent during hospital visits by patient and caregivers incur heavily to national and global economic development. WHO estimates that the disease burden requires up to 3.5 billion US dollars extra per year, on top of the current outrageous budget, to fill the resource gap in implementing existing TB interventions (73).

Policies for childhood TB management are now available after many years of limited attention to pediatric TB challenges mainly because they are known not to transmit the disease widely in the community (22). Children are seen as less of a risk than adults mainly because of paucibacillary nature of their disease, but also because it is harder to diagnose them. It is therefore clear that TB is an important contributor to maternal and childhood morbidity and mortality.

This was further emphasized in the Moscow declaration to end TB, at the ministerial conference, where age-related social and health inequalities were observed and thus, consequently, commitments made to prioritize children among high-risk groups and populations in vulnerable situations that critically need urgent attention in order to achieve TB elimination (74).

1.3 Tuberculosis diagnosis

1.3.1   Microbiological diagnosis

The evolution of TB diagnostics tests has seen a turn of events in development of more advanced techniques in recent years. However, none of them has been able to fulfill the global ideal target of a simple, rapid and affordable, yet reliable test for diagnosis of all forms of TB. Some of the traditional methods continue to play a significant role in many high burden low resource settings mainly because of their simplicity but are not very reliable especially in paucibacillary samples common in HIV infected and young people. Others have good accuracy and reliability but require complex systems including laboratory infrastructure and biosafety concerns that are not easily available in most resource limited settings. More work is still needed in order to meet the global priorities for TB care and control which comprise of improving early case-detection, including cases of smear-negative disease that are often associated with HIV coinfection and young age, and

to enhance the capacity to diagnose MDR-TB (70). The WHO- End TB Strategy calls for early diagnosis of TB and universal drug-susceptibility testing to guide appropriate treatment, highlighting the critical role played by laboratories in the TB management and control. In line with that, Foundation for Innovative New Diagnostics (FIND) also recommends simpler, more robust and easy-to-use tests (75) with hope to achieve the global goal of ending TB. Below are some of the diagnostic methods that are currently in use around the world.

1.3.1.1 Smear microscopy

Smear microscopy is one of the oldest methods of diagnosing pulmonary TB. There are two commonly used microscopy methods with sensitivity and specificity differences. These methods continue to suffer at the expense of new technology mainly because of inter reader variability concerns coupled with poor sensitivity and inability to differentiate viable from non-viable organisms, which is a limitation for its monitoring of treatment response.

Ziehl Neelsen (ZN) microscopy: Also known as conventional light microscopy, ZN technique uses stained smears prepared directly from sputum specimens. In most high burden countries, detection of acid fast bacilli (AFB) in a smear is sufficient to declare a confirmed TB (76). For this reason, ZN has been the primary diagnostic test for TB and remains a useful method in most low resource settings. One of the advantages is that it can be used without stable electricity but requires at least a concentration of 10,000 organisms/ml (77) in a sample for a test to be positive. However, the sensitivity is highly variable with lowest and highest reported at 20% and 80% respectively, as compared to mycobacterial culture (78). With such low sensitivity in some facilities, this is a major limitation of the test especially in young children and HIV infected populations where most samples are majorly paucibacillary (79).

LED/ fluorescence microscopy
Thanks to the use of light emitting diode bulbs, fluorescent microscopy could be used with or in replacement of ZN microscopy, taking advantage of its better sensitivity and faster reading(78,80,81). The use of battery operated LED with robust attachments on the already existing microscopes became convenient in resource-limited settings. Accuracy data on LED

microscopy shows pooled sensitivity and specificity of 84% and 98% against culture as reference standard (78). Based on this evidence, WHO released a new policy on the use of LED based FM for diagnosis of TB emphasizing the operational and cost benefits over conventional FM and ZN techniques (78).

1.3.1.2 Mycobacterial culture

For many years, TB culture was a gold standard test for TB diagnosis, DST and treatment monitoring but is constrained by long turnaround time. In most settings, the most common methods include liquid media like Mycobacteria Growth Indicator Tube (MGIT) and solid media like Lowenstein Jensen (LJ). Samples from none sterile sites for MGIT or LJ culture must undergo decontamination mostly using Nalc-NAOH method to remove other micro-organism in order to allow growth of MTB. Despite being the gold standard tests MGIT and LJ are constrained by requirement for high infrastructure that is rarely available in most low resource settings. In addition, these tests require technical expertise and biosafety standards. For that reason, culture has been centralized in most of these countries mainly for DST on specific referred samples from the peripheral health facilities.

LJ medium is an egg-based enriched medium containing glycerol, asparagines, malachite green among others. The principles behind culture media is that the ingredients L-Asparagine and potato flour are sources of nitrogen and vitamins in LJ medium (82). Monopotassium phosphate and magnesium sulfate enhance organism growth and act as buffers while glycerol and the egg suspension provide fatty acids and proteins required for the metabolism of mycobacteria. The coagulation of the egg albumin during sterilization provides a solid medium for inoculation purposes while sodium citrate and malachite green are selective agents to prevent growth of most contaminants and allow early growth of mycobacteria. LJ can detect viable bacilli of 100 bacilli per milliliter compared to direct microscopy which requires 10000 bacilli and above; per milliliter and permits preliminary differentiation of mycobacteria on the basis of colony morphology while providing the necessary material for biochemical identification and drug susceptibility testing. LJ

is most widely used in low resource countries, but requires up to 8 weeks to declare a negative result. One of its advantages is that it is easy to prepare and can be made locally. It also has an advantage of allowing colony count in a semi-quantitative approach. The semi-quantitative tests are important in studies evaluating in vitro bacteriocidal concentrations for certain MTB strains.

MGIT was developed by Becton Dickinson in 2006 as a replacement for the conventional BACTEC 460 that was deemed risky because of using radioactive carbon 12, sharp needles and glass. The MGIT system relies on the ability to exploit the fluorescence of an oxygen sensor to detect growth of mycobacteria in culture(83). MGIT was evaluated against the precursor conventional BACTEC 460 medium with excellent results, especially when used in combination with a solid media (84,85). The MGIT tube contains Middlebrook 7H9 liquid media, and an oxygen-quenched fluorochrome, tris 4, 7-diphenyl-1, 10-phenonthroline ruthenium chloride pentahydrate, embedded in silicone at the bottom of the tube. This oxygen sensitive sensor is dissolved in the broth and when bacteria grow within the tube, they utilize the free oxygen that is replaced with carbon dioxide. With depletion of free oxygen, the fluorochrome is no longer inhibited, resulting in fluorescence within the MGIT tube that is visualized under UV light. The intensity of fluorescence is directly proportional to the extent of oxygen depletion. Growth can also be detected visually by the presence of a non-homogeneous turbidity or small grains or flakes in the culture medium. MGIT is supplemented with essential components to enhance rapid growth of mycobacteria. These include oleic acid, with an important role in the metabolism of mycobacteria; albumin, as a protective agent that binds free fatty acids that maybe toxic; dextrose, an energy source; and catalase that destroys toxic peroxides that may be present in the medium. At the time of positivity (usually 7-14 days) if MTB is present, the number of bacilli in the medium is approximately $10^5 – 10^6$ colony forming units (CFU) per ml of medium. The instrument declares a tube negative if it remains negative for six weeks (42 days)(83). One of the advantages of using MGIT is that it can detect low quantity of bacilli in a sample compared to LJ. One evaluation study reported the lowest detection threshold of less than 10 organisms using the MGIT 960 liquid culture with a turnaround time of 5-22 days(86). MGIT culture is more sensitive than LJ. From the accuracy data reported in a meta-analysis the sensitivity and specificity of MGIT was 81.5%

and 99.6% as compared to a composite culture based reference standard of BACTEC 960/MGIT, BACTEC 460TB and solid media, respectively(87). Because MGIT is highly enriched, growth of contaminating bacteria is usually common. For that reason, addition of antimicrobial mixture called PANTA™ (Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin) helps to reduce contamination rate close to that generally experienced with solid media (83).

1.3.1.3 Molecular tests

All the Nucleic Acid Amplification (NAA) for drug resistance testing have been developed based on principles of whole genome sequencing. Despite the old history of molecular methods, in most developing countries, these tests are a relatively new thing in the TB diagnosis with the initial methods relying on classical in-house principal that required complex manipulations(88). These methods have now been simplified by automating the different steps of the NAA or with the use of strips. NAA are rapid with a sensitivity approaching that of culture methods.

The Xpert MTB/RIF (Xpert) assay using the GeneXpert platform (Cepheid, Sunnyvale, CA, USA) is a typical example of a molecular method that simplifies microbiological detection of organisms. It is based on nucleic acid amplification that fully integrates and automates three processes required for real-time PCR testing; specimen preparation, amplification and detection (Figure 4). Xpert detects MTBc and its mutations for rifampicin resistance using three specific primers and five unique molecular probes to ensure a high degree of specificity (70). The assay allows processing of results directly from sputum in less than 2 hours (89). This has a lot of significance in resolving the issue of single day testing that was affecting patients' results uptake and treatment. With sufficient quality of sputum, this test can be highly accurate and reliable with sensitivities of approximately 98% and 72% among smear-positive and smear-negative samples and specificity of 99.2%(90). This analytic sensitivity is equivalent to detection of as few as 131 cfu/ml of MTB spiked into sputum. The Xpert technology is based on molecular beacons that target the *rpoB* gene that covers all the mutations found in more than 99.5% of all rifampicin-resistant strains. The method does not require a biosafety level three, as normally applied in culture, considering that the sample reagent used kills more than 6 log10 cfu/ml (97%) of MTB within 15

minutes of exposure, and does not generate infectious aerosols during inoculation and testing. Similarly, high sensitivities and specificities were reported in detecting rifampicin resistance; 97.6% and 98.1% and with a mean time to detection of less than 1 day (90).

Figure 5: Xpert MTB/RIF system. Source: Cepheid website, 2018

In a systematic review and meta-analysis of pulmonary TB in children, Xpert pooled sensitivity and specificity from respiratory samples was 62% (95% CI 51-73) and 98% (97-99), respectively (91). With this method, concerns related to delayed-testing, as seen with other less sensitive methods that required next day visit to provide early morning sample, resulting in high costs to the patients, were reduced. Based on the above quality of evidence, WHO issued policy recommendations on the use of Xpert as the initial diagnostic test in adults and children suspected of having TB and MDR-TB or HIV-associated TB (70).

The roll out of Xpert MTB/RIF was highly successful and approximately 34.4 million cartridges so far procured for TB diagnosis by 133 of 145 countries eligible for concessional pricing(6). At

the same time, some of the 22 high burden countries still rely on microscopy testing but recent data shows an improvement in 2015 compared to 2014(92).

As shown above, one of the recurrent concerns has been the low detection accuracy of Xpert in smear negative samples, besides the requirement for power, and maintenance costs. Hence, the Xpert has since been modified to cater for better sensitivity and better uptake. This has led to development by Cepheid of the Xpert Ultra cartridge. Its sensitivity is much closer to culture especially among paucibacillary samples(93,94) while reducing turnaround time even further. Xpert ultra can detect as few as 13 cfu/ml of MTB in sputum. Xpert Ultra has a sensitivity of 88% vs 83% for Xpert MTB/RIF assay in adults and a specificity of 96% vs 98%(95). Accuracy data in children show sensitivity and specify of 62% (51-73) and 98% (97-99) respectively(91). In addition the manufacturer has developed simple battery operated platform known as Xpert Edge (figure 5) to facilitate its use in primary health settings.

Figure 6: Genexpert edge. Source: Cepheid website

Loop-Mediated Isothermal Amplification (TB LAMP) is a unique, temperature-independent technique for amplifying DNA. It is simple to use, providing a visual display that is easy to read yet robust and can be used at peripheral health centres, where microscopy is performed. Originally used for malaria, this commercial molecular assay was developed by Eiken Chemical Company (Tokyo, Japan) to detect MTB based on LAMP technology. Unlike other molecular assays, this method is manual, requires less than 1 hour to perform and can be read with a naked eye using ultraviolet light. The TB-LAMP method operates under similar biosafety standards as smear

microscopy. In addition, it is easy to use, offers fast diagnosis and requires minimal infrastructure, and can be placed at primary health level with potential to replace the less sensitive smear microscopy test (96).

A systematic review in adults reported a sensitivity for TB-LAMP ranging between 77.7- 80.3% (96). One of the disadvantages is that this method cannot identify cases of rifampicin resistance. This limits its operational benefits in countries with high burden of rifampicin resistance.

Childhood data was not evaluated but the recommendations are extrapolated in children based on the generalization of data from adults.

Thus based on this evidence, WHO recommended that TB-LAMP may be used as a replacement or follow-on test for sputum-smear microscopy to diagnose pulmonary TB in adults with signs and symptoms consistent with TB (96).

1.3.1.4 Urine LAM

The urine lateral flow urine Lipoarabinomannan Assay (LF-LAM); Abbott Laboratories, Lake Bluff, USA (formerly Alere Inc, Waltham, USA) test is the first commercialized antigen-based point of care test endorsed by WHO for diagnosis of TB. The test is based on detection of urine LAM antigen, a lipopolysaccharide present in mycobacterial cell walls, which is released from metabolically active or degenerating bacterial cells. The test is simple and resourceful in patients that cannot produce sputum or those with paucibacillary samples.

Unfortunately, its sensitivity remains low with estimates from meta-analysis reporting between 13% - 93%, while specificity from 87% to 99% using microbiological confirmation as gold standard (97). The sensitivity and specificity is increased in HIV infected patients, in particular those with low CD4 count: 37% (16–62) and 100% (81–100) for CD4 <200, 35% (14–62) and 100% (94–100) for CD4 between 50-100 (97). The urine LAM is also a prognostic test with several studies reporting increased mortality in HIV infected patients with a positive urine LAM results (98). WHO recommended the use of this test under two categories: i) in persons with HIV infection and low CD4 counts or who are seriously ill, ii) in HIV positive adult in-patients with signs and

symptoms of TB who have a CD4 cell count less than or equal to 100 cells/µL, or HIV positive patients who are seriously ill, but should never be used as a screening test (99).

There is still need for further scientific evidence on the test among different populations including children. In a prospective study among HIV and non HIV infected children with presumptive TB in South Africa, urine LAM test had a poor accuracy against reference standard with sensitivity and specificity of 48.3%% (37.6-59.2) and 60.8% (56.1-65.3) respectively(100).

1.3.1.5 Other diagnostic tests

Serological testing for TB: Serological tests have been used successfully to diagnose most infectious diseases in the past. They are based on the principle of antigen-antibody reactions that can be used to detect infectious diseases. One of their advantages is that they use blood that is easy to collect in all age groups and do not require high infrastructure and biosafety measures. Because of these benefits, attempts have been made to use serology to detect MTBc but with serious difficulty. Several individual studies have demonstrated highly variable sensitivity and specificity. In the WHO-TDR program evaluation of several serological rapid commercial tests, in comparison with composite standard of culture plus clinical follow-up, similar variability in sensitivity values was observed ranging from 1- 60% and specificity of 53- 99% (101). All these evaluations have been performed among adults and there is no data in children.

Thus WHO strongly recommended that these commercial tests not be used for the diagnosis of pulmonary and extra-pulmonary TB (101).

Breathalyzer: This is a non-invasive TB point of care test that is believed to offer rapid screening at a low-cost. The breathalyzer (Rapid Biosensor Systems Ltd) test is simple, based on detection of actively infectious MTB antigen (Ag85B) that can be coughed out as aerosols in sputum and tested using immune-sensor and bio-optical technology (102,103). It does not require extensive infrastructure and can potentially be used by non-medical staff. One of the advantages of this test is that it can diagnose TB in patients who do not produce sputum and can detect disease during

very early stages. In addition, there is no infection control concern since the closing of the tube effectively seals the Breathalyzer and protects the user from contact with the sample.

Field evaluations show a sensitivity ranging from 74-94% when the breathalyzer antigen test is combined with smear microscopy with a relatively low specificity (79%) and a good tolerability (102). This test is still under evaluation and is not yet endorsed by WHO for diagnosis of TB.

Interferon-Gamma Release Assays (IGRAs): These measure the presence of immune reactivity to MTB in an exposed person. White blood cells of infected persons release interferon-gamma when mixed with antigens derived from MTB. The test uses fresh blood samples that are mixed with antigens and controls. One of the challenges faced with this test is that it does not differentiate LTBI from active disease. Two tests have been approved by the U.S. Food and Drug Administration and these are commercially available: QuantiFERON®-TB Gold In-Tube test (QFT-GIT) and T-SPOT®.TB test (T-Spot).

In one of the recent evaluations, the sensitivity and specificity of the QFT-GIT assay for active TB were 84% (95% CI, 70-93) and 70% (95% CI 61-79), respectively. The IFN-γ/TNF-α-dual release assay by fluorospot had substantially higher diagnostic specificity (94%) for diagnosing active TB than the IFN-γ-single release assay (72%, $p < 0.001$), without compromising sensitivity (84% vs. 89%, $p = 0.79$) (104). There is limited data on accuracy of IGRA in children and its role in predicting active disease progression.

Hence, CDC recommends that IGRAs can be used in place of (but not in addition to) TST in all situations in which CDC recommends TST as an aid in diagnosing MTB infection, with preferences and special considerations including: contact investigations, testing during pregnancy, and screening of health care workers and others undergoing serial evaluation for MTB infection (105).

1.3.2 Sample collection

Microbiological confirmation is the ideal standard for TB diagnosis but requires that laboratory diagnostic tests are performed on good quality samples. The most commonly used respiratory

sample, sputum needs to be properly collected if the results are to be accurate and reliable. However, it is not always easy to obtain sputum especially in younger children. For that reason, several alternative methods have been proposed and evaluated using different diagnostic tests. In this chapter, we describe some of the commonly used sample collection methods and their challenge for TB diagnosis in children.

1.3.2.1 Sputum

For many years, sputum sample has been used to diagnose TB. The microscopy, molecular methods and culture all rely on a good quality sputum sample in order to provide a definitive diagnosis. Getting quality sputum samples require a productive cough and good explanation on how to collect the samples. False-negative TB test result may result from poor quality sputum specimen, such as salivary specimen. Therefore, patients should be explained how to produce a good specimen and asked to produce another one if the specimen is of poor quality(106). Morning specimens are usually of better quality but may raise some operational constraints for patients who need to bring these specimen the next day and this can increase the risk of patients dropping out during investigations(106). Even from the same patient, the positivity of laboratory tests is often variable between specimen(79). Indeed that was the earlier justification for collecting and testing more than one sputum sample from each person with presumptive TB (107).

With more evidence, the adequate number of samples for a proper diagnosis was established through observational studies. In a retrospective meta-analysis of data from presumptive TB patients, a combination of samples was tested to identify intra sensitivity of 1-3 samples on microscopy and culture. An incremental yield of 8.4% was reported for the second specimen and 3.5% for the third specimen (108). All sensitivity rates were higher among patients with pulmonary cavitation but was generally low with the microscopy method. For microscopy, WHO currently recommends same day diagnosis using two consecutive sputum specimens ('spot-spot') examined on the same day (so-called 'front-loaded' or 'same-day') diagnosis(109). However, with the introduction of more sensitive rapid diagnostic tests than smear-microscopy such as Xpert, one sputum sample is enough for TB diagnosis of patients with presumptive TB(70).

To improve the incremental yield from sputum and reduce the laboratory workload and cost of repeated tests, it has been proposed that samples could be pooled in order to increase the concentration of bacilli before molecular or culture testing. Discordant results were reported regarding the effect of pooling samples on the detection yield and so far this approach is not recommended, requiring more evidence(103, 107).

Children, especially below 10 years represent a complex group that has difficulties to spontaneously provide sputum. Because of that, a number of sampling options have been proposed to help in diagnosis of pulmonary TB. Each of the options has strength and weakness and getting a universal sampling method for children remains a challenge to TB diagnosis.

1.3.2.2 Gastric aspiration

Gastric aspiration (GA) is a classical alternative specimen collection method for patients unable to produce sputum, including children. The method collects gastric liquid in the stomach that contains bacilli swallowed during sleep or through coughing or muco-ciliary clearance. Although gastric acid in the stomach is expected to inhibit growth of most microorganisms, mycobacteria by nature of their cell wall are able to partially or temporarily withstand these conditions; and require processing for culture or DNA within 4 hours. Because of this GA samples must be neutralized with sodium bicarbonate in children with intrathoracic TB to maintain viability. The use on neutralizing buffer to reduce contamination is not universally accepted as a remedy for reducing contamination as emerging data shows contradicting outcomes in samples treated with sodium bicarbonate (111). In the WHO broad evaluation, Xpert on GA samples showed a pooled sensitivity of 83.8% among adults and children using culture reference standard (70). Among children with presumptive TB, detection yield from GA ranged between 1 and 45% for culture and 5 and 50% for Xpert across studies in a systematic review (112). The challenge with this method is that it is an invasive procedure that is not well tolerated because of need for prolonged fasting, besides inconveniences related to hospitalization and infection control risks (113). Some studies have shown that this method is reliable and can be used among ambulatory and hospitalized

patients (114). However, in routine practice, this method is poorly utilized outside reference hospitals in many settings. This is probably due to lack of infrastructure because of resource constraints in most poor settings.

Another challenge with GA is potentially the high risk of culture contamination from NTM from digestive tract (115).

1.3.2.3 Induced sputum

Induced sputum is another sampling method based on the induction of the sputum by inhalation of hypertonic saline through nebulization combined by nasopharyngeal aspiration in young children. It is known to induce a small amount of airway secretion that can be expectorated and analysed(116). There is belief that the increased osmolality of the airway lining fluid rises vascular permeability in the bronchial mucosa and encourages production of mucus by submucosal glands(116).

Despite a potential risk of bronchospasm that requires to have oxygen and bronchodilator available when the procedure is performed, induced sputum is generally well tolerated (117). Minor side-effects are coughing, epistaxis, vomiting, or wheezing. One of its advantages is that it has no age restriction and can be applied successfully in children. Among the challenges is that it requires nebulization and suction facilities that are not always available in most low resource setting and the potential infection control risk of transmission to health care workers and other patients through the aerosols generated requiring that the procedure is performed in a dedicated well ventilated area, which can be challenging in some facilities. Although the procedure is considered of lower TB transmission risk and low cost (118), specimen collection in children is curtailed by infrastructure and system challenges that have limited the use of induced sputum implementation in most primary health facilities. Besides the collection challenges, induced sputum has a good detection yield with most laboratory testing methods. In a recent systematic literature review on diagnosis of childhood intrathoracic TB, culture yield from induced sputum ranged between 1-30% and Xpert 3-17%(112).

1.3.2.4 Broncho-alveolar lavage

Broncho-alveolar lavage (BAL) through the introduction of a flexible bronchoscope into a sub-segment of the lung involves instillation of sterile normal saline into a sub segment of the lung, followed by suction and collection of the instillation for analysis. It is supposed to be the most effective method because it collects alveolar sample. This procedure requires high experienced clinician and high technical platform. It is expensive and impractical in most resource poor settings. It is also an invasive procedure that requires specialized training and is not well tolerated in children.

1.3.2.5 Nasopharyngeal aspiration

Nasopharyngeal aspiration (NPA) is a more simple method based on the insertion of a graduated suction catheter through the nostril into the oropharynx to stimulate a cough reflex with secretions aspirated mechanically. Childhood data on the use of NPA is sparse and more evidence is needed before it can be recommended by WHO as one of the specimen collection methods in children. The few field studies have reported varying performances with Xpert. one study reported a slightly lower sensitivity of 44% than sputum induction; 60% (119) while others reported good accuracy among HIV infected population (120). In a recent systematic review, among children with presumptive TB NPA had a detection yield of 4-24% with culture and 3- 8% with Xpert (112). Others have reported better sensitivity that was higher using smear and culture for NPA than the IS in other studies (121). The method is well tolerated with low risk of bronchospasm than the IS, is operationally more feasible than the GA and can be easily implemented in ambulatory setting. It however requires aspirator and mucus extractor that can be challenging to purchase in low resource countries. It is therefore a very promising method for specimen collection in children.

1.3.2.6 Stool sample

More recently, stool has become a lucrative sample as an alternative to sputum for diagnosis of TB in patients unable to produce sputum including children. The mechanism that discharges MTBc

in stool is now known as patients swallow the viscous sample either intentionally or unintentionally during sleep resulting in deposition of a rich stool sample. The only major challenge with stool sample has been the overwhelming number of artifacts that may inhibit the PCR reaction requiring specimen processing before Xpert testing.

Most importantly, the PCR inhibitors have been shown to degrade nucleic acid assays including the Xpert test. Emerging data on Xpert on stool using different processing methods (concentration or floatation methods) from a recent meta-analysis indicate varying sensitivities (67%, 95%CI 52 to 79%) and specificities (98 to 99%) with a very high heterogeneity(122). Because of this variation and laborious nature of the procedures, attention is currently being put on optimizing the processing methods. Therefore, this specimen is not yet recommended by WHO for diagnosis of TB.

1.3.2.7 Other samples

Other samples are also important to consider especially for diagnosis of extra-pulmonary TB that can represent up to 30% of all TB forms(123). Depending on the site, different samples may be considered for laboratory diagnosis. Peripheral lymphadenitis is the most frequent extra thoracic form of TB disease manifestation usually affecting the neck region with much risk among children between 1-10 years(124). Lymph nodes usually have large concentrations of bacilli and are therefore the best for capturing TB in children as they usually exhibit characteristic features associated with the disease. These lymph nodes contain several bacilli that have been phagocytically contained during immunological response. Usually a painful swelling of one or more lymph nodes occurs. Most often, the disease is localized to the anterior or posterior cervical chains in approximately 70-90% of cases or supra clavicular. It is often bilateral and non-contiguous. Diagnosis can be done using biopsy of the lymph node or just a fine needle aspiration of the lymph node followed by Xpert testing or culture. Other samples include cerebral spinal fluid in cases of disseminated TB, pleural fluids and more recently urine, among others. In the field accuracy evaluation of Xpert on extra-pulmonary samples, sensitivity was 83.1% (71.4-90.7) in lymph nodes, 80.5% (59-92.2) in cerebral spinal fluid, 46.4% (26.3-67.8) in pleural fluids, with

specificities above 98%(125). Xpert from urine among patients with urinary tract TB has also been investigated with sensitivity of 94.4% (81.3–99.3) and specificity of 89.5% (85.1–92.9) against culture and could be considered for diagnosis of disseminated TB(126).

Oral swabs: these have also come up as potential alternative samples. Interestingly, a good sensitivity has been reported in different sites of the mouth. In a South African study, two different swab brands were used to collect samples from three sites within the oral cavity. The study observed that tongue swabbing using a flocked swab yielded better signals on the PCR than cheek or gum swabbing. A combined sensitivity of 92.8% relative to sputum Xpert was observed using two tongue swabs per patient. The specificity of the Oral swab was 91.5% (127). The cause of most false-positive results were reportedly due to contamination of manual PCRs. Hence, oral swabs could potentially work as alternative sample facilitate TB diagnosis in clinical settings and patient like children who are challenged by sputum collection.

1.3.3 Sample transportation

Transportation of samples to testing facilities is very important in influencing the outcome of laboratory investigations. In most laboratory procedures, a fresh sample is desirable. However, most facilities with expertise and technology to perform better diagnosis are far from the primary health centres. According to specific instructions from the manufacturer, specimens to be tested on Xpert should be held at 2-8 °C for 10 days maximum or be stored at a maximum 35°C for up to 3 days before processing while for culture it should be processed immediately or kept at 2-8 °C not beyond 3 days (128).

It is therefore necessary to preserve samples using cold chain that is hard to achieve in such settings because of unreliable power and faulty or inaccessible equipment like fridges and freezers (to prepare icepacks). For this reason, simple, reliable and cost effective sample preservative methods that are compatible with most common diagnostic tests would be convenient for low resource settings. Commercial products that preserve the viability of TB bacilli and reduce bacterial contamination in patient specimens at the point of collection or, when added to specimens after receipt at the central laboratory, may aid in the recovery of mycobacteria or preserve the bacterial

DNA for molecular testing(129). These may eventually bridge the gap between sampling and final diagnosis thereby guiding better clinical judgement.

Some of the available methods are described in this chapter.

1.3.3.1 Cetyl-pyridinium chloride

Cetyl-pyridinium chloride (CPC) is an ammonium compound that has antiseptic and bactericidal effect on most microorganisms. This chemical has been adopted to preserve samples that have delayed access to the laboratory for culture. One of the advantages of CPC is that it is cheap, easy to use and effectively inhibits growth of unwanted microorganisms. In addition, samples preserved with CPC can be decontaminated virtually during shipment thus reducing human resource time and consumables. For these reasons, CPC is commonly added to samples during storage or transport for partial digestion and decontamination (130). It has been in use for many years because of its low toxicity to MTB but also due to the decontamination effect on bacteria and other normal flora from the mouth (131). Several evaluation studies, from different countries, have been done in the past to confirm the effect of this compound to MTB. Most of these studies tested these properties at ambient temperature for several days (131,132). Others have also compared CPC recovery of MTB from clinical samples of pulmonary TB patients against standard N-acetyl L-Cystine (NALC) - sodium hydroxide decontamination method (NAOH) (133).

Remarkably, CPC was found to have a higher recovery of MTB than the standard method particularly in paucibacillary samples. Indeed all studies evaluating the culture yield of CPC against the standard NALC-NAOH method have reported significantly better outcomes with the CPC preservative in samples tested after long delays (134–136). Studies on use of CPC treatment before Xpert testing reported good performance as well with results comparable with those of culture (135). The only problem has been its utility to reproducibly enhance MTB growth in liquid media. One study reported a 15% reduction in MGIT culture growth among smear positive samples treated with CPC compared to non-treated counterparts (137).

Considering that most samples from the field require both LJ and MGIT, the lack of compatibility option has led to limited utilisation of CPC globally.

1.3.3.2 Ethanol

Historically, ethanol has been known to preserve DNA for molecular biology work mainly because of the killing effect on potential agents while protecting DNA quality. One of the advantages of ethanol is that it is widely available and less costly in most countries. The effect of ethanol fixation on PCR detection and viability of MTB in human sputum sediments was previously evaluated (138). The study used sputum samples seeded with MTB and achieved effective killing within one hour signifying the strength of ethanol at 50-95% concentration. The study demonstrated that ethanol fixation of human sputum sediments significantly reduces the risk of exposure to MTB and provides a good transport medium for clinical specimens for DNA assays.

Similarly, ethanol has been used as a safety measure to fix MTB smears for microscopy before staining. The concentration, exposure time and amount of organic material is very important determining the potency of ethanol. In one study, the effect of 70% ethanol was found not sufficient after 5-10 minutes of exposure hence suggesting that the bacilli may still be transmissible(139). Unfortunately, ethanol cannot be used with culture.

With these many benefits of ethanol, field evaluations are necessary especially in the era of Xpert in many facilities, to systematically establish the length of time that this reagent can preserve sputum samples collected from known TB patients in order to guarantee reproducibility in different context.

1.3.3.3 OMNIgene® SPUTUM

OMNIgene® SPUTUM (OM-S) is another commercial sample preservation method that recently became available. It builds on the successes and weaknesses of CPC preservation methods in order to provide options to sputum preservation. It is expected that OM-S can be used in places where refrigeration and cold chain conditions are unavailable yet delayed specimen transportation to culture facilities is inevitable. Using the same model as for CPC, studies have looked at the ability OM-S to preserve samples longer without cold chain. Preliminary studies evaluated the reagent in a resource-limited setting and reported a significantly reduced culture contamination, and improved TB detection on LJ medium compared to standard NALC-NAOH methods (140).

Unlike CPC, the beauty of OM-S has been shown by its compatibility with liquid medium in isolating MTB from clinical samples. Studies conducted in various parts of the world show that it is possible to harvest similar TB proportions from liquid and solid medium using OM-S that is comparable with standard NALC-NAOH method (141–143). The only concern raised in these studies is the delay in turnaround time as MTB is detected slightly longer within the OM-S group. This is promising considering the long standing challenge that was limiting liquid culture methods. More evidence has recently come from FIND confirming the compatibility of OM-S with MGIT and improved results compared to standard NALC-NAOH method (129).

However, there is a growing concern as compatibility results of OM-S with MGIT cultures reported in the above studies cannot be reproduced in other settings. Two recent studies have shown that on the contrary, despite high ability to suppress contaminants, and improve recovery of MTB in LJ cultures, OM-S interferes with MGIT cultures rendering them falsely negative (144,145). This indicates that further optimization of OM-S is need for samples requiring MGIT cultures with the aim of improving results and to reduce the turnaround time. Besides culture, preliminary and subsequent assessments with smear and Xpert test reported good performance that was comparable with samples treated under cold chain or fresh samples (142,146).

Manufacturers of OM-S recommend that the reagent can preserve samples up to 30 and 8 days for Xpert and culture respectively(140). With this promising concordance already reported, this could

be instrumental in programmatic settings where Xpert sites and surveillance centers require that samples are inevitably delayed before testing. Some studies have compared the compatibility of all the three reagents described; Ethanol, CPC, OM-S with molecular diagnostic testing methods among preserved sputum samples. They reported excellent results with CPC and OM-S but slightly lower yield with ethanol especially among low grade smears(147).

With these contradictions, in results by different groups, more data is needed to guide universal utilisation of this reagent.

1.3.4 Paediatric TB diagnosis

Microbiological confirmation of TB is the best way to confirm TB disease. This requires collection of appropriate samples. Unlike adults, the natural history and clinical manifestations in children make it complex to rely on microbiological confirmation.

1.3.4.1 Challenges of paediatric TB diagnosis

Under routine practices, only a small proportion of children with TB disease are confirmed microbiologically. Indeed, even those children that get successfully screened, the inability to produce a suitable sample to offer confirmed diagnosis is a big limitation. With difficulty, children generally cannot easily expectorate and often produce smaller quantities of sputum compared to adults. In most cases, sputum in children is usually swallowed making it difficult to obtain good quality sputum samples to perform laboratory tests to confirm TB diagnosis.

Another limitation is the paucibacillary nature of pulmonary TB in children as compared to adults that makes the existing TB diagnostic tests from respiratory samples less performant in children than in adults(124). Thus despite availability of diverse diagnostic methods as described above, availability of a suitable sample remains key.

Because of that, the majority of children on TB treatment are started "empirically" based on the TB contact history, clinical presentation and chest X-ray findings. There are many challenges related to clinical diagnosis of TB in children because they tend to present with characteristics that are non-specific and often mimic those of other common disease conditions such as HIV, pneumonia and malnutrition (148).

Also, the clinical presentation varies with different stages of disease progression and the typical symptoms or signs of TB like unremittent cough, failure to thrive, weight loss, reduced playfulness; low-grade or intermittent fever are less frequently present in the early stage (149). Later with respiratory involvement, the most common clinical symptom is a persistent, non-remitting cough or wheeze that is unresponsive to antibiotic treatment for other agents that usually show nonspecific signs for TB (150).

Worse still, this lack of specificity is more pronounced in children with other comorbidities like HIV or severe malnutrition (151). It is therefore a big challenge for non-experienced staff to base on symptoms and easily triage children and later on diagnose them with TB. This may result in a risk of under or over diagnosis. Under diagnosis is the major problem that results in untreated children who may develop severe form of the disease at a later date. Many of these children are likely to be managed for other conditions especially pneumonia that strongly mimics TB disease. Exposing children to unnecessary drugs further complicates the clinical conditions.

Over diagnosis is less a problem except for the use of unnecessary 6 month course of poly-antibiotic treatment with potential toxicity coupled with the risk of not treating another pathology that could have mimicked TB. A recent meta-analysis has reported that up to 23% of children admitted with clinical presentation of severe pneumonia in high TB burden countries may suffer from TB and that the case fatality rate of these severe pneumonia associated to TB may be up to 20% (152).

Without a suitable sample, options like chest X-ray become critical. Unfortunately, radiological findings of TB in children may also lack specificity and are often difficult to be recognized by non-trained health personnel. Interpretation of chest X-ray in children has some specificities as

compared to adults, that may induce wrong interpretation and over treatment for tuberculosis, such as the confusion of a persistent thymus with mediastinal adenopathy.

In addition to the lack of training of staff, most primary health centres and even secondary health centres in some limited resource countries do not have X-ray or intermittent access due to power-cut and shortage of reagents. The interpretation of the X-ray is always hampered by the poor quality of the films due to lack of maintenance, poor supply of reagents and lack of training of radiographers on pediatric chest-X ray, in particular regarding the need of lateral X-ray (124).

Besides the lack of X-ray, the access to this test is limited by the fact that most national TB programs only cover the cost of sputum smear or Xpert testing and that of treatment but rarely for the X-ray. As a result, most children tend to receive empirical treatment only based on clinical presentation without chest x-ray. Indeed, in most countries the management of pediatric TB remains too much concentrated at tertiary or secondary health care level with primary health care only referring for diagnosis.

Operational costs due to transport coupled with poor road network in most low resource settings are a significant barrier to the referral and results in many children with presumptive TB who are not investigated for TB. Very little attention is given to primary health care to develop their capacity to diagnose and treat pediatric TB, even in centres treating adults. In addition, there is limited guidance on how, when and where children should be screened for TB (153).

Very often children stand a higher chance of getting infected from contact with sick adults in the household (154). The poor implementation of screening of child contacts is another limitation to early detection of TB in children (155). Indeed this can be shown by the low IPT coverage as only approximately 13% of eligible children below 5 years receive preventive therapy (8). Thus it is commendable that symptom based screening of any child contacts of an adult diagnosed with TB should be done at facility or even in the household in order to promptly identify children requiring further investigations and offering IPT to asymptomatic children.

Taking into account the poor performance of microbiological diagnosis and poor access to chest X-ray in resource-limited countries, WHO recommends using algorithmic approach for diagnosis of tuberculosis in children (22) as shown in Figure 7:
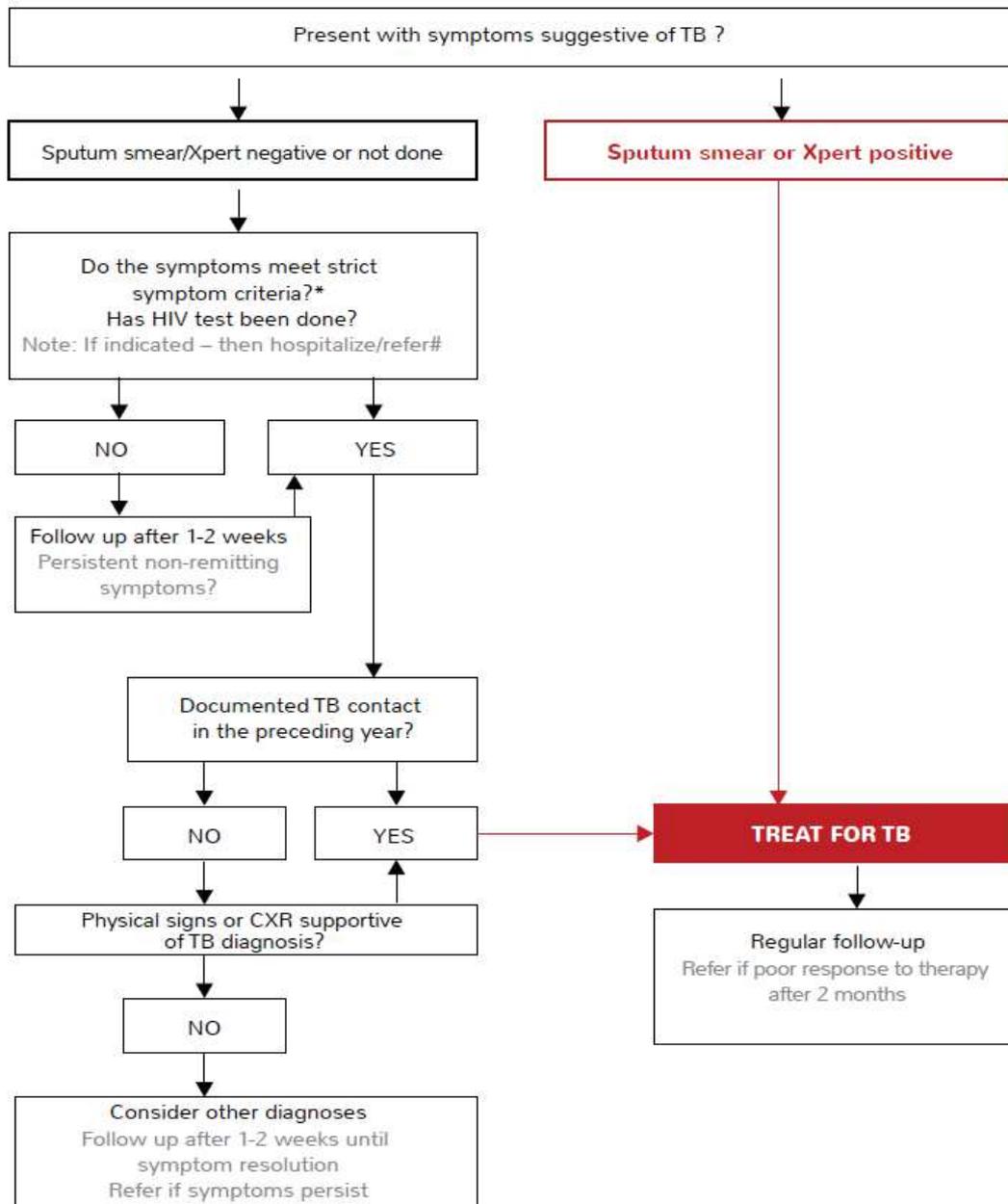
Figure 7: Guidance for the diagnosis of children who present with symptoms suggestive of TB.
Source: WHO 2014

1.3.4.2 Lack of reference standard

Finally, there is currently lack of a suitable reference standard for diagnosis of intrathoracic TB in children that constraint the evaluation of new diagnostic tests in children. Although culture from respiratory samples remains an imperfect reference standard, it will identify an important part of the pulmonary TB in adults. In children, a significant part of intrathoracic TB will not be detected by culture from respiratory sample for all the reasons listed above. Unfortunately, microbiological confirmation is possible in only 15%–50% of pediatric TB cases (156).

The lack of a good reference standard for diagnosis of TB in children partially explains the lack of studies that evaluate new diagnostic tests for paediatric TB. To overcome these challenges, experts have proposed using a proxy-composite reference standard based on well-defined standard case definition taking into account clinical, radiological, history, microbiological and outcomes with or without TB treatment and review by an independent panel of experts.

According to the revision of these case definitions published in 2015, children with presumptive TB are then classified in three categories of confirmed TB, unconfirmed TB and unlikely TB as shown in the algorithm of Figure 8 and Table 1 (156).

Table 1: Summary of intrathoracic TB case definitions for diagnostic evaluation studies in children (156).

Case Definition	Refined Criteria[a]
Confirmed tuberculosis	Bacteriological confirmation obtained Requires *Mycobacterium tuberculosis* to be confirmed (culture or Xpert MTB/RIF assay) from at least 1 respiratory specimen

Unconfirmed tuberculosis	Bacteriological confirmation NOT obtained AND at least 2 of the following:
	
	• Symptoms/signs suggestive of tuberculosis (as defined)
	
	• Chest radiograph consistent with tuberculosis
	
	• Close tuberculosis exposure or immunologic evidence of *M. tuberculosis* infection
	
	• Positive response to tuberculosis treatment (requires documented positive clinical response on tuberculosis treatment—no time duration specified)
	
	  - With *M. tuberculosis* infection
	    • Immunological evidence of *M. tuberculosis* infection (TST and/or IGRA positive)
	  - Without *M. tuberculosis* infection
	    • No immunological evidence of *M. tuberculosis* infection

Unlikely tuberculosis	Bacteriological confirmation NOT obtained AND
	Criteria for "unconfirmed tuberculosis" NOT met
	
	  - With *M. tuberculosis* infection
	    • Immunological evidence of *M. tuberculosis* infection (TST and/or IGRA positive)
	  - Without *M. tuberculosis* infection
	    • No immunological evidence of *M. tuberculosis* infection

Abbreviations: IGRA, interferon-γ release assay; TST, tuberculin skin test.

[a] *All children should have symptoms compatible with tuberculosis as determined by the treating clinician.*
Source Graham et al., 2015
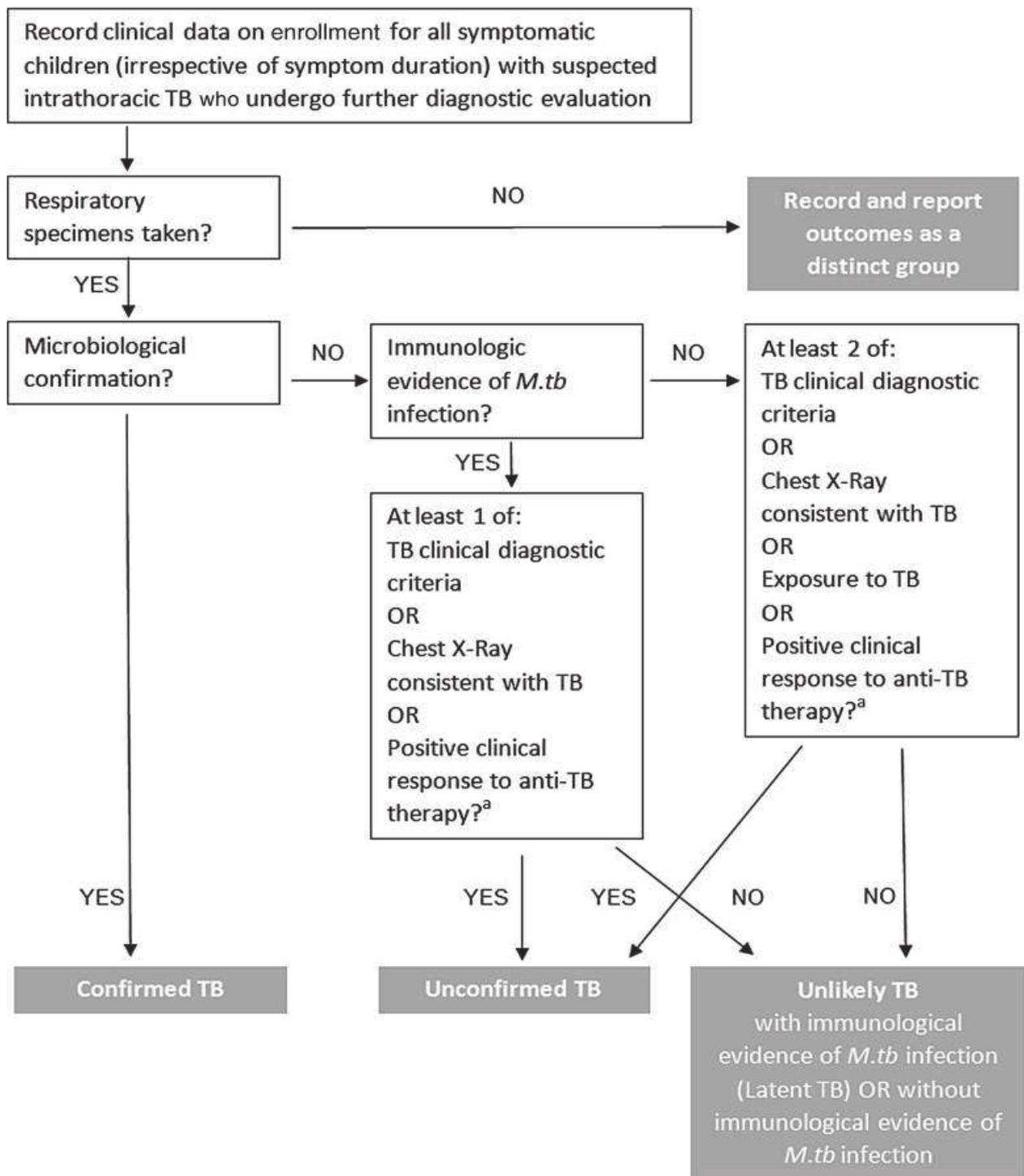
Figure 8: Recommended diagnostic algorithm for paediatric TB. (Source: Graham et al., 2015)

CHAPTER 2

JUSTIFICATION, OBJECTIVES AND METHODS

2.1 Justification

Among the reasons of poor bacteriological diagnosis of intrathoracic childhood tuberculosis, the challenge of obtaining sputum from children has been well described. Unlike adults, where sputum collection is not a major problem, children have difficulty producing sputum and are left with limited options. Of particular interest in overcoming this challenge are samples that may be easy to collect from children while offering accurate and reliable diagnosis. Fortunately, the robust mycolic layer of MTB organism can resist the rigorous environment in the stomach, particularly the hydrochloric acid, allowing the bacilli to remain viable in stool for laboratory investigations. Although, gastric aspirate has shown to be a reliable sample for diagnosis of TB in children, due to operational challenges limiting its implementation in low resources settings, further consideration was given to the stool samples. At the same time, urine is very simple to collect and is routinely easily available. Mycobacteria antigens such as the Lipoarabinomannan have been found in this sample especially in highly immunocompromised persons and a point of care test (Urine LAM) is already commercialized and recommended by WHO for diagnosis of tuberculosis in HIV advanced infected patients. This means that both urine and stool, if found reliable and offering accurate diagnosis, would be important for children and other age groups where similar challenges prevail. There is currently limited evidence on the use of stool or urine samples to diagnose pulmonary TB in children. We believe that the findings from this project will contribute significantly in adding more evidence on the use of these samples while also highlighting the magnitude of paediatric TB burden in our setting. This will guide policy makers on planning and allocating reasonable resources in the management of paediatric TB in Uganda.

In addition to sample collection, sample management and transportation in many low resource countries is a critical concern. There are still many challenges in achieving cold chain in the field as well as during transit to laboratories where appropriate diagnostic technology is placed. Some countries, including Uganda, rely on post office deliveries that have improved the transport systems but still take long to get samples to final destination because of infrastructural difficulties. This often leads to breakdown of cold chain on transit, and also pose an infection control risk. It remains important to have preserving solutions that can keep samples longer without cold chain yet viable for a long time across different temperature and humid conditions. Such reagents or

compounds that would allow treatment of samples uniformly across different laboratory procedures including Xpert and culture on solid or liquid media would be very useful. There is contrasting data from different preservatives. In this study we proposed to evaluate a new promising preservative (Omnigene) and an old and cheap reagent (ethanol). The combination of child-friendly specimen collection method that could be deployed at low level of heath care facilities where most children seek care and of good preservative to transport samples without cold chain to reference laboratory for testing. It is expected to improve the quality of diagnosis of pediatric tuberculosis in high burden and low resource countries.

2.2 Objectives

General Objectives

The main objective is to improve the diagnosis of tuberculosis in children from a high TB-HIV prevalence and low resource setting.

Specific objectives

- To evaluate the performance of Xpert assay for diagnosis of intrathoracic childhood tuberculosis from respiratory and stool samples among children with presumptive tuberculosis.
- To assess the outcomes of children with presumptive tuberculosis.
- To evaluate the performance of Xpert from stool and urine LAM for diagnosis of tuberculosis among children admitted with severe illness
- To explore field sputum sample preservation methods that can support delayed testing on Xpert and MGIT culture.

2.3 Materials and methods

2.3.1 Study site

2.3.1.1 Mbarara

This project includes three diagnostic studies conducted within Mbarara, southwestern Uganda, at a regional referral hospital and a research facility belonging to Epicentre.

Mbarara Regional Referral Hospital (MRRH) is found in Mbarara district, west of Kampala the capital city of Uganda (Figure 8). It is a public hospital funded by the government of Uganda through the Ministry of Health (MoH). It is a regional referral hospital for South Western Uganda serving 10 districts with an estimated population of more than 5 million people. The Hospital also receives patients from the borders of neighboring countries such as Rwanda, Tanzania and Democratic Republic of Congo. According to the most recent census report, Mbarara district alone has a population of 474,144 of which 229,901 are females (157). MRRH is also a teaching hospital for the Medical School of Mbarara University of Science and Technology (MUST). Children with presumptive TB are referred from the paediatric outpatient department to the paediatric TB clinic which runs once a week for both HIV-infected and HIV-negative children. At the time of the first study implementation in 2009, hospital records indicated that there were 75 children in the year started on TB treatment and among these, 30% were HIV-TB co-infected. Records indicated that mean age was 58 months (5 months -17 years) and 70% of children were younger than 5 years. The hospital facility has a paediatric ward with a nutritional facility to support malnourished children. It also has an isolation unit for children identified with TB.

Figure 9: Map of Uganda showing Mbarara district: https://www.unicef.org/uganda/ug_2010_districts.jpg

2.3.1.2 Epicentre

Epicentre is an epidemiological and research non-governmental organization attached to Médecins Sans Frontières. Since 1999, it has a research center embedded in the MUST nearby the MRRH. The research agenda of Epicentre is guided by health related needs of the community but with a major focus on influencing policy in line with global and regional relevant objectives. Epicentre conducts studies according to Good Clinical Practices (GCP) and Good Clinical and Laboratory Practices (GCLP).

The centre has close collaborations with the MUST, the MRRH, the Ministry of Health and other stakeholders in research, academia and the health sector. Current projects continue to focus on improving diagnosis and therapies for TB, HIV, central nervous system infections, as well as vaccine trials on Ebola and Yellow fevers. The Mbarara research center includes a biosafety level 3 laboratory equipped to perform microscopy, Lowenstein and MGIT culture, Xpert and line probe assay.

The laboratory is part of the national laboratory network in Uganda under the stewardship of the Uganda supra-national reference lab. In addition, the laboratory is supervised by the Supra national Reference Laboratory at the Institute of Tropical Medicine, Belgium and participates in the proficiency scheme of National Health Laboratory Services (NHLS), South Africa for microscopy, Xpert and culture. The laboratory also performs hematology and biochemistry analyses to address the above disease burden. These are also included in the proficiency scheme with the NHLS.

2.3.2 Study design and population

This project presents data from two pediatric studies and one adult study that were conducted at MSF-Epicentre Mbarara Research Centre, Uganda.

In the first study, children with presumptive TB aged between one month and 14 years were enrolled into a prospective diagnostic study. All enrolled children in this cohort had received a comprehensive TB assessment comprising of clinical, radiological assessment, smear microscopy, MTB culture, Xpert on respiratory and non-pulmonary specimens including stools for diagnosis of tuberculosis. Children were prospectively followed regardless of the TB diagnostic decision on or off anti-tuberculosis treatment that allows to describe the outcomes of children according to treatment decision.

The second study was built based on results from the first diagnostic study that highlighted high mortality among children with severe illness or comorbidities, especially among those whose microbiological confirmation of TB was not possible. Therefore, we proposed a prospective diagnostic study enrolling admitted children with severe illness or comorbidities such as severe malnutrition and HIV infection that were associated with poor outcomes in the first cohort. Two non-sputum based diagnostic tests, Xpert MTB/RIF assay from stool and urine LAM were evaluated against microbiological and composite reference for confirmed and unconfirmed TB using uniform case definitions, respectively.

The third study was a laboratory proof of concept study that explored two specimen preservation, using Omnigene (DNA Genotek, Canada) and ethanol to determine the possibility to store sputum

up to 30 and 8 days respectively before performing Xpert and MGIT culture, without affecting the TB detection yield. For this study, adults with confirmed respiratory TB from the smears were enrolled and samples collected for the evaluation. These were split into aliquots that were tested head to head for the different test methods.

CHAPTER 3

RESEARCH STUDIES

Chapter 3.1

Xpert diagnosis of childhood tuberculosis from sputum and stool samples

3.1.1 Justification and objectives

The use of sputum to diagnose TB in children is now a widely conceived challenge. This has greatly affected notification rates as less than 50% is reported annually. It is believed that, as described in the previous chapters, one of the challenges is associated with poor diagnostic options. The lack of capacity to produce sputum, greatly undermines progress made already with the current diagnostic methods. By using stool as an alternative to respiratory samples, we believe that children could easily receive appropriate TB diagnosis and increase detection rates globally. At the same time, as there is limited data on paediatric burden in this region, findings from respiratory samples would help to explain the situation by establishing paediatric TB data that would help in addressing some of the questions related to childhood TB diagnosis. Although the estimated burden of childhood TB is expected to be approximately 10-20% in such a high TB-HIV burden country, more data is need to form a baseline for future studies(158).

Standard case definitions were recently proposed to be used as a composite reference standard in the context of paediatric TB research(159). This reference standard needs further applications in different settings in order to justify its generalizability. We believe that evidence from this study will improve childhood TB diagnosis and treatment outcomes in the region. In addition, data from this project is expected to open new research questions, especially for the evaluation of new diagnostic interventions in children that could be addressed in the near future.

Objectives:

- To document the proportion of microbiologically confirmed TB in children with presumptive TB by using different microbiological diagnostic tests and by age groups.
- To assess the performance of Xpert MTB/RIF on sputum and stool in diagnosing TB among children with presumptive TB.
- To explore the potential benefit of pooling sputum samples in order to reduce the laboratory workload, turnaround time and cost of Xpert MTB/RIF testing.

3.1.2 Methods, results and conclusion

Method:

This was a prospective observational study of children with presumptive tuberculosis; defined as presence of at least one clinical sign suggestive of TB or TB contact history with an abnormal chest X-ray or any child with a chest X-ray suggestive of TB. All children with at least one sign of presumptive TB aged between one month and 14 years presenting at MRRH received a comprehensive and standardized clinical and biological evaluation. Biological assessment included smear microscopy, MTB culture (Lowenstein-Jensen and MGIT), Xpert, on two sputum or induced sputum for children unable to expectorate for diagnosis of TB. Xpert and culture were performed after pooling of respiratory samples to reduce laboratory workload. At the stage of proof of concept, all children with a decision to start TB treatment by the clinician with or without microbiological confirmation had two stool samples collected for Xpert testing.

Performance of Xpert from respiratory samples were assessed against a culture-based reference standard from sputum and a composite reference standard using the published standard case definitions of intrathoracic childhood tuberculosis from 2012(159). Performance of Xpert from stool was assessed against Xpert and/or culture from sputum and against a composite reference standard. Children without indication of TB treatment had a systematic clinical assessment after 3 months and those started on TB treatment were followed up to 6 months after completion of treatment. For the classification of children using the reference standard, digital chest X-ray was read by 2 independent radiologists or expert clinicians and clinical files from all children with presumptive TB were retrospectively reviewed by 2 independent paediatric TB experts in order to classify the cases as Confirmed, Probable, Possible, Unlikely and Not TB using the 2012 standard case definitions for intrathoracic childhood TB(156).

Results

Sample collection in this study was generally well implemented as 91% of children produced at least one sputum. The quality of sputum sample collected was not affected by the age of the child. Despite good sputum collection and exhaustive assessment, we only microbiologically confirmed TB in 4.3% (17/392) of children in this cohort. Although we used three testing methods (microscopy, Xpert and culture), the highest proportion of TB detection was obtained with Xpert (4%) and mainly among children above 10 years of age. Indeed, performance of Xpert on sputum was comparable with culture with a sensitivity and specificity of 90.9% (58.7-99.8) and 99.1% (97.4-99.9), respectively.

We attempted to test the added benefit of combining sputum samples in order to increase the TB detection potential of microscopy or culture while reducing workload. Overall, there was no added value in pooling sputum samples together.

Furthermore, close to half of the children started on TB treatment offered stool samples for the analysis. Among these, 11.3% were microbiologically confirmed using Xpert or culture. Against the microbiological reference standard, the sensitivity and specificity of stool Xpert was 55.6% (21.2-86.3) and 98.2% (90.3-100).

Conclusion

The proportion of confirmed TB does not reflect the expected prevalence considering that a large number 36.7% (144/392) of these children were started on TB treatment. It also does not match the estimates from such a high TB/HIV burden country where rates of 10-20% would be expected (158). Secondly, the quality of respiratory samples collected was good despite the variable age groups in this study. This may be influenced by the fact that this was a controlled research environment where the health workers are well trained to give adequate instructions during sample collection. It has been previously shown that with good instruction sample collection improves tremendously (106). In terms of pooling samples to obtain a better yield, this study did not support this extra processing step as there was no added value observed as it has been recently observed in a related study in South Africa (110).

Sputum xpert was comparable to culture. This adds more evidence on the importance of Xpert in the diagnosis of pulmonary TB as previous studies have reported good but slightly less performance of the test (91).

Stool Xpert had an average sensitivity and excellent specificity in this study. This is much related to what has been documented by other groups (160–162). The possible explanation for the low sensitivity could be due to low bacilli in the stool samples that could have been lost through the processing methods.

3.1.3 Involvement in this work

In this study my involvement was mainly in the protocol development and study implementation. As laboratory manager, I coordinated development of analytical plans, procedures and other document that were used to capture data from the laboratory. In addition, I supervised the laboratory activities to ensure that they were performed according to the good clinical and laboratory practice as well as laboratory quality management system. I took lead in data analysis and manuscript writing and submission to the appropriate journal. Finally, the manuscript was published in the European journal of clinical microbiology. Full publication is shown in next page.

ORIGINAL ARTICLE

CrossMark

Xpert MTB/RIF diagnosis of childhood tuberculosis from sputum and stool samples in a high TB-HIV-prevalent setting

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Abstract

The Xpert MTB/RIF assay is a major advance for diagnosis of tuberculosis (TB) in high-burden countries but is limited in children by their difficulty to produce sputum. We investigated TB in sputum and stool from children with the aim of improving paediatric TB diagnosis. A prospective cohort of children with presumptive TB, provided two sputum or induced sputum at enrolment in a regional referral hospital in Uganda. Stool was collected from those started on TB treatment. All specimen were tested for Xpert MTB/RIF, mycobacteria growth indicator tube (MGIT), Lowenstein Jensen cultures and microscopy (except stool). We compared TB detection between age categories and assessed the performance of Xpert MTB/RIF in sputum and stool. Of the 392 children enrolled, 357 (91.1%) produced at least one sputum sample. Sputum culture yield was 13/357 (3.6%): 3/109 (2.6%), 3/89 (3.2%), 3/101 (2.6%) and 4/44 (8.2%) among children of < 2, 2–5, ≥ 5–10 and > 10 years, respectively ($p = 0.599$). Xpert MTB/RIF yield was 14/350 (4.0%): 3/104 (2.9%), 4/92 (4.3%), 3/88 (2.9%) and 4/50 (.0%), respectively ($p = 0.283$). Sensitivity and specificity of Xpert MTB/RIF in sputum against sputum culture were 90.9% (95% CI 58.7–99.8) and 99.1% (99.1–99.8). In stool, it was 55.6% (21.2–86.3) and 98.2% (98.2–100) against Xpert MTB/RIF and culture in sputum. Only a minority of children had microbiologically confirmed TB with a higher proportion in children above 10 years. Although sensitivity of Xpert MTB/RIF in stool was low, with good optimization, it might be a good alternative to sputum in children.

Keywords Childhood tuberculosis · Stool · Sputum · Xpert MTB/RIF

Introduction

Childhood tuberculosis (TB) diagnosis continues to be a major challenge. To date, there is no optimum diagnostic test in children and their exact TB burden remains unknown [1, 2].

In a 2017 WHO report, it was estimated that approximately 1 million children below 15 years developed TB in 2016 but only 45% were notified [3]. This could be explained by the fact that that current diagnosis of TB in children relies on a thorough assessment of evidence derived from a careful history of exposure, clinical examination, tuberculin skin test and chest X-ray when available, and rarely bacteriological confirmation [4, 5]. Because of limited bacteriological confirmation, it is possible that in the absence of an expert opinion, clinical judgement could be misleading as it is mostly subjective. In the absence of non-sputum-sensitive diagnostic tests, microbiological tests rely on examination of respiratory sample but are limited by difficulty in obtaining a sputum specimen in children and the paucibacillary nature of childhood intrathoracic TB [6].

Based on a recent meta-analysis, the sensitivity (95% confidence intervals) of the Xpert MTB/RIF as compared to that of culture is 62% (51–73) for sputum or sputum induction and 66% (51–81) for gastric lavage or aspirate with specificities of 98% (97–99) and 98% (96–99) respectively [7]. However, Xpert MTB/RIF is still constrained by difficulty in using sputum induction, or gastric aspirate or lavage, in health facilities of limited resource settings [8]. This has influenced a lot of interest in the use of stool specimen to detect TB bacilli from

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respiratory aspirates that are swallowed for the diagnosis of childhood TB. Currently, there is still limited data on the performance of Xpert MTB/RIF on stool with studies reporting varying sensitivities and specificities [8–12].

There is need for more supportive data on stools as potential suitable replacement to sputum testing in children unable to produce sputum, and development of sensitive and feasible diagnostic tests for TB in children living in high-burden and low-resource settings remains a priority.

The aims of this study were (1) to document the proportion of microbiologically confirmed TB in children with presumptive TB by using different microbiological diagnostic tests and age groups, (2) to assess the performance of Xpert MTB/RIF on sputum and stool in diagnosing TB among children and (3) to explore the potential benefit of pooling sputum samples to reduce the laboratory workload, turnaround time and cost of Xpert MTB/RIF testing.

Methods

Study design and population

This was a prospective observational study of a cohort of children in Uganda aged 1 month to 14 years with presumptive TB defined as the presence of at least one clinical sign suggestive of TB or TB contact history with an abnormal chest X-ray or any child with a chest X-ray suggestive of TB. Both out- and inpatients were enrolled at Mbarara Regional Referral Hospital, Southwestern Uganda. For stool evaluation, only children started on TB treatment were enrolled and the performance of the MTB/RIF on stool used the standard case definitions of childhood intrathoracic TB as reference standard [13].

Study procedures

A paediatrician interviewed parents/guardians regarding symptoms and TB contact history and examined the child.

Sample collection and laboratory analysis

Sputum sample Children were requested directly or through their parents or guardian to give two sputum samples on spot and early morning the next day. Children unable to produce sputum had an option of sputum induction using 15 ml of 5% hypertonic saline nebulized for up to 20 min if sufficient sputum (at least 2 ml) was not obtained before. In younger children, suctioning through the nasopharynx or oropharynx was done using a sterile mucus extractor.

Within 24 h of collection, the two samples were first processed separately for smear microscopy and the remaining samples were then pooled and decontaminated and corresponding

sediment was split. One part was tested with Xpert MTB/RIF and the other with culture. Smear microscopy was performed using auramine staining and Olympus microscope equipped with the FluorescenS® LED system (magnification, × 20–40) for reading. The number of acid-fast bacilli (AFB) in 100 high-power fields (HPF) was graded using the WHO/IUATLD AFB microscopy grading scale [14]. External laboratory quality assurance was based on proficiency panels from National Health Laboratory Services, South Africa.

Pooled sputum specimen was decontaminated using the N-acetyl-L-cysteine–sodium hydroxide (NALC-NaOH) method (1.5% final concentration). It was then concentrated by centrifugation at 3000 relative centrifugal force (RCF) for 20 min at 4 °C. The pellet was re-suspended in phosphate buffer saline (PBS). Two drops (100 µl) of re-suspended sediment were inoculated into each of the two Lowenstein Jensen (LJ) medium and 0.5 ml into mycobacteria growth indicator tube (MGIT) supplemented with PANTA (polymyxin B amphotericin B nalidixic acid trimethoprim azlocillin cocktail) and OADC (oleic acid albumin dextrose and catalase supplement). For LJ culture, results were qualitative (i.e. positive, negative). The average of number of colonies on all the LJ tubes per specimen was reported following the WHO grading scale [14]. For MGIT culture, inoculated tubes were read daily using manual MGIT fluorescence reader. Cultures (MGIT and LJ) that remained negative after 8 weeks of incubation were classified as negative. Those that turned positive were proceeded with Ziehl Neelsen microscopy to check for AFB and blood agar culture to assess the possibility of contamination. Cultures with AFB were confirmed for *Mycobacterium tuberculosis* complex using MPT 64 (SD Bioline)-Rapid Diagnostic Test. The laboratory is controlled by the supranational TB laboratory of the Tropical Medical Institute of Antwerp (Belgium).

Besides the culture, the sediment from the decontaminated sample was used for Xpert MTB/RIF testing according to instructions in the package insert. In brief, 500 µl of sediment was mixed with 1.5 ml sample treatment buffer, shaken vigorously and incubated for 15 min at room temperature before it was transferred to the Xpert MTB/RIF cartridge [15].

Stool sample Apart from sputum, all children with a decision by a paediatrician based on exposure, clinical, radiological and bacteriological findings to start TB treatment had one stool sample collected, tested within 24 h with culture and Xpert MTB/RIF. Stool was processed prior to Xpert MTB/RIF testing to remove polymerase chain reaction (PCR) inhibiting factors. Decontamination was based on modified protocol described by Walters et al., 2017 [8]. A sample was homogenised with saline solution, vortexed in the screw-capped container for 10 s and left to stand for 5 min. After settling, 5 ml of supernatant was picked and transferred into a 50-ml centrifuge tube. An equal volume of NaOH-NALC

solution (1.5% final concentration) was added, vortexed lightly and left to stand for 20 min. PBS was added up to the 50-ml mark, centrifuged for 20 min each at $3000 \times g$ and 4 °C [8]. To reduce the possibility of contamination, the decontamination step was repeated on the sediment. After centrifugation, the supernatant was poured off and 1.5 ml of buffer was added to re-suspend the pellet. Of this, 0.5 ml was inoculated into MGIT supplemented with OADC and double-strength PANTA, two drops into each of the two LJ tubes and 0.5 ml to prepare for Xpert MTB/RIF testing [15].

Retrospective classification of tuberculosis cases

In addition to the onsite clinician, postero-anterior and lateral digital chest X-rays were read by an external radiologist who was blinded to the child's clinical presentation. All patients' files were retrospectively reviewed by two independent senior paediatricians in order to classify each case as confirmed, probable, possible, unlikely and not TB using the 2012 published standard case definitions for intrathoracic childhood TB [13].

Statistical analysis

Consecutive children with presumptive TB were recruited over 2 years. Data were entered using Voozanoo (Epiconcept, Paris, France) and analysed using Stata® 13 software (College Station, Texas, USA). Baseline children's characteristics (demographic, medical history, TB exposure, clinical, radiological) were summarised using percentages for categorical variables, and medians and interquartile ranges (IQR) for continuous variables. Sample collection and bacteriology results were presented by four age groups (< 2 years, ≥ 2 to < 5 years, ≥ 5 to < 10 years, ≥ 10 years) [16].

MTB detection yield using Xpert MTB/RIF and different culture methods was compared between pooled sample and individual samples (when patients were not able to produce two samples to be pooled).

Sensitivity, specificity and predictive values of the Xpert MTB/RIF assay in sputum (pooled or individual) were evaluated against culture (MGIT and LJ) in sputum after exclusion of Xpert invalid results and culture contaminated or positive culture with growth of non-tuberculosis mycobacterium (NTM) without MTB. Any MTB-positive culture defined a positive reference standard and at least one negative culture result without any positive MTB culture result defined a negative reference standard. Sensitivity, specificity and predictive values of Xpert MTB/RIF and culture in stool were evaluated using culture or Xpert MTB/RIF results from sputum after exclusion of Xpert MTB/RIF invalid/culture contaminated or culture results with NTM growth without MTB growth. A positive reference standard for the stool evaluation was any

culture or Xpert MTB-positive sample, and a negative reference standard was at least one negative result without any MTB-positive result. When using the standard intrathoracic case definitions as reference standard, confirmed and probable TB cases defined a positive reference standard, and possible, unlikely and not TB cases as negative reference standard.

Median (interquartile range) of time to treatment initiation was calculated from the date of the first sample collection to the date of treatment initiation for children that started on anti-TB treatment.

Results

A total of 392 children presumed to have TB disease were enrolled between April 2012 and January 2014 (Fig. 1). Of these, 178 (45.4%) were females, 125 (31.9%) were below 2 years of age and 121 (31.0%) were HIV infected (Table 1). Three hundred and fifty-seven (91.1%) and 285 (72.7%) produced at least one and two sputum samples respectively (Table 2). For children that produced at least one sputum sample, those aged ≥ 5–10 years had the highest collection yield.

Overall, microscopy detected AFB from five (1.4%) children. After exclusion of contaminated results and cultures with NTM, sputum culture confirmed TB in 13/349 (3.7%) of the patients. The culture detection yield was more than twice higher in the group of children ≥ 10 years old (4/48, 8.3%) compared to other age groups (9/301, 3.0%), $p = 0.088$. After exclusion of invalid results, Xpert MTB/RIF confirmed TB in 14/348 (4.0%) patients with also a higher yield among older children (4/50, 8.0% and 10/298, 3.4%), $p = 0.126$.

The total number of microbiologically confirmed TB cases (Xpert or culture) was 17/392 (4.3%) in all patients and 17/357 (4.8%) in children with at least one sputum collected. Out of these, MTB was detected in 13/17 (76.5%), 8/17 (47.7%) and 14/17 (82.4%) by LJ, MGIT and Xpert MTB/RIF, respectively. LJ media had an incremental yield of 5/13 (38.5%) patients in sputum compared to MGIT alone (Table 3), and both LJ tubes were positive except in one sample that had contamination in the second (data not shown). Similarly, of 15 cases detected either by culture or by Xpert MTB/RIF in sputum, 10 (66.7%) were detected by both methods, 4 (26.7%) by Xpert MTB/RIF only and 1 (6.7%) by culture only (Table 4). Xpert MTB/RIF sensitivity and specificity in sputum were 90.9% (58.7–99.8) and 99.1% (97.4–99.9) (Table 4). A total of 144 children were started on TB treatment. The median (IQR) time to treatment from first sample collection was 2 days (0, 3).

As shown in Table 5, pooling two sputum samples did not increase the TB detection yield of Xpert MTB/RIF, LJ and MGIT culture.

Fig. 1 Study profile
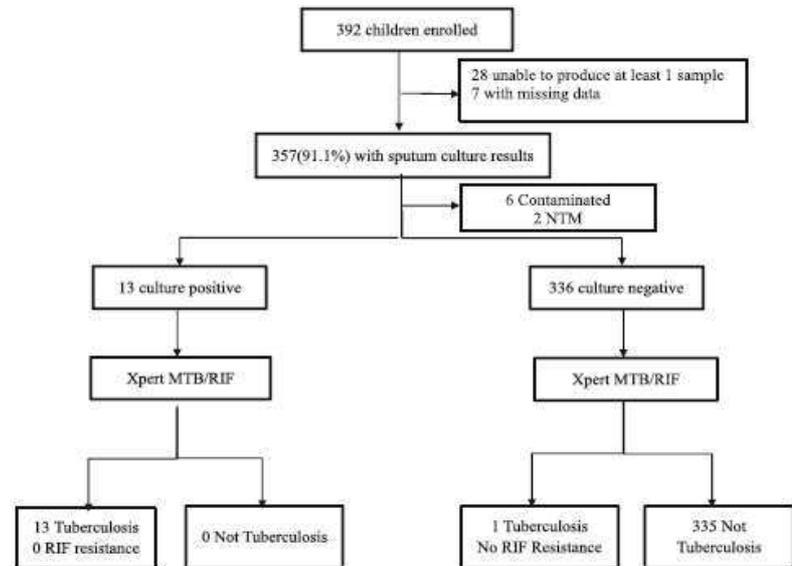
Table 1 Patients' characteristics at inclusion

Characteristics	$N = 392$
Sex, female, n (%)	178 (45.4)
Age, n (%)	
< 2 years	125 (31.9)
2–5 years	103 (26.3)
5–10 years	112 (28.6)
> 10 years	52 (13.3)
HIV status, n/N (%)	
Positive	121/388 (31.2)
CD4 cell count, median [IQR] ($N = 27$)	418 [203–988]
On ART	70/121 (57.8)
BGC vaccination, n/N (%)	338/349 (96.8)
TB contact history, n/N (%)	76/391 (19.4)
Previously treated for tuberculosis, n (%)	8 (2.0)
Received antibiotics 2 weeks before inclusion, n/N (%)	182/270* (67.4)
Clinical findings, n/N (%)	
Cough > 2 weeks	361/387 (93.3)
Reported fever for 7 days	145/392 (37.0)
Night sweats > 2 weeks	103/392 (26.3)
Unexplained weight loss	185/392 (47.2)
Unexplained fatigue > 2 weeks	137/392 (35.0)
Tuberculin skin test, n/N (%)	
< 5 mm	284/383 (74.1)
≥ 5 mm	99/383 (25.8)
Chest X-ray, n/N (%)	
Normal	131/382 (34.3)
Abnormal TB suggestive	138/382 (36.1)
Abnormal non-TB suggestive	113/382 (29.6)

IQR, interquartile range; *BCG*, Bacille Calmette-Guérin vaccine; *TB*, tuberculosis

*Data collected secondarily after protocol amendment

Table 2 Mycobacteria tuberculosis detection among different age categories in respiratory samples

	Overall	< 2 years	2 to < 5 years	≥ 5 to < 10 years	≥ 10 years	p
Sputum collection, N	392	125	103	112	52	
≥ 1 sample, n (%)	357 (91.1)	109 (87.2)	93 (90.3)	106 (94.6)	49 (94.2)	0.168
2 samples	285 (79.8)	87 (79.8)	72 (77.4)	84 (79.2)	42 (85.7)	
1 sample	72 (20.2)	22 (20.2)	21 (22.6)	22 (20.7)	7 (14.3)	
Microscopy results, N	355	109	92	105	49	0.201
Negative, n (%)	350 (98.6)	109 (100)	90 (97.8)	104 (99.0)	47 (95.9)	
Positive, n (%)	5 (1.4)	0	2 (2.2)	1 (1.0)	2 (4.1)	
Culture results, N	357	109	94	105	49	0.599
Negative, n (%)	336 (94.1)	102 (93.6)	89 (94.7)	101 (96.2)	44 (89.8)	
Contaminated, n (%)	6 (1.7)	3 (2.8)	2 (2.1)	0	1 (2.0)	
MTB, n (%)	13 (3.6)	3 (2.6)	3 (3.2)	3 (2.6)	4 (8.2)	
NTM, n (%)	2 (0.6)	1 (0.9)	0	1 (1.0)	0	
Xpert MTB/RIF, N	350	104	92	103	50	0.283
Negative, n (%)	334 (95.4)	100 (96.2)	88 (95.7)	100 (96.1)	46 (92.0)	
MTB, n (%)	14 (4.0)	3 (2.9)	4 (4.3)	3 (2.9)	4 (8.0)	
Invalid, n (%)	2 (0.6)	1 (0.9)	0	1 (1.0)	0	

MTB, Mycobacterium tuberculosis; NTM, non-tuberculosis mycobacteria

Seventy-one stool samples were tested with Xpert MTB/RIF and culture among the 144 children (49.3%) started on treatment, because majority did not produce stools during the observation time. Of them, eight (11.3%) were TB confirmed by either Xpert MTB/RIF or culture on stool: three by both methods, four by Xpert MTB/RIF only and one by culture. Compared to sputum culture or Xpert MTB/RIF reference standard, the stool Xpert MTB/RIF sensitivity and specificity were 55.6% (21.2–86.3) and 98.2% (90.3–100), respectively (Table 6). There was one stool sample positive by Xpert MTB/RIF but negative by the reference standard. However, by using standard case definitions as proxy reference standard that could be applied to 69/71 patients, 26 patients were classified with a positive reference standard (9 confirmed and 17 probable TB cases) and 43 as negative reference standard. After exclusion of two cases with invalid Xpert results, sensitivity, specificity, positive and negative predictive values of Xpert MTB/RIF in stool were 7/26 (26.9%, 11.6–47.8), 41/41

Table 3 Concordance of different culture media in respiratory samples

MGIT (n)	Lowenstein Jensen medium (n)				
	Contaminated	Negative	MTB	NTM	Total
Contaminated	11	42	1	0	54
Negative	7	282	4	1	294
MTB	0	0	8	0	8
NTM	0	1	0	0	1
Total	18	325	13	1	357

MGIT, Mycobacterium growth indicator tube; MTB, Mycobacterium tuberculosis; NTM, non-tuberculosis mycobacteria

(100%, 91.4–100), 7/7 (100%, 64.6–100) and 41/60 (68.3% 55.0–79.7), respectively. Of the seven cases with MTB detected in stool, four (57.1%) were HIV infected, one (14.3%) had severe malnutrition and two (28.6%) had TB contact history with one of them presenting with cavities on chest X-ray.

Discussion

Despite the use of exhaustive TB diagnostic methods and alternative respiratory specimen collection (sputum induction), only 17/392 (4.3%) of children with presumptive TB were microbiologically confirmed. This proportion is lower than what has been reported in previous diagnostic studies from Sub-Saharan Africa using culture from induced sputum (15–30%) or gastric aspirate (6–32%) [9, 17–22]. The low pre-test probability of TB in our study might explain the low detection. Indeed, TB suspicion was based on the presence of at least one TB suggestive symptom or sign, which was different from what was used in other studies that based on two symptoms or signs. In Zambia, using at least one TB suggestive sign to define presumptive TB, 6.2% of Xpert MTB/RIF detection yield was MTB/RIF [23].

As expected, the lowest proportion of TB detection yield was in children below 5 years due to the paucibacillary nature of TB, coupled with difficulties in obtaining a suitable respiratory samples [6]. It has also been associated with the wide spectrum of disease manifestations that often overlap with other common childhood conditions such as pneumonia, HIV-associated lung disease and malnutrition [24–27].

Table 4 Performance of Xpert MTB/RIF in sputum against sputum culture

	Sputum culture			
	MTB	Negative	Contaminated	NTM
Xpert MTB/RIF				
MTB	10	3	1	0
Negative	1	325	5	2
Invalid	0	2	0	0
Sensitivity	10/11 (90.9% 58.7–99.8)			
Specificity	325/328 (99.1% 97.4–99.8)			
Positive predictive value	10/13 (76.9% 46.2–95.0)			
Negative predictive value	325/326 (99.7% 98.3–100)			

MTB, *Mycobacterium tuberculosis*; *NTM*, non-tuberculosis mycobacteria

Surprisingly, out of 15 confirmed TB samples, 3 were Xpert MTB/RIF positive and culture negative despite the fact that all our patients were newly diagnosed. In addition, 10% of culture-confirmed cases were Xpert MTB/RIF negative, which is lower than what has been reported in previous meta-analysis of Xpert MTB/RIF in children [7]. We could not independently investigate this in our current study. However, we believe that possibly some of these children were taking fluoroquinolones as part of the antibiotic course for respiratory infections prescribed before admission. This could have resulted in dead bacilli detected by Xpert MTB/ RIF but not growing in culture. A similar scenario has been previously reported in an adult study in the same setting [28]. In addition, at a final concentration of 1.5%, the NaOH decontamination procedure could have been harsh for the already paucibacillary samples since it is known to kill at least 30% of the TB population [29, 30].

Finally, the MGIT culture which is known to be more sensitive than LJ was unexpectedly underperforming in this study [31, 32]. There was no explanation for this except that double-strength PANTA in MGIT could have inhibited MTB in some paucibacillary samples and that we performed two LJ cultures

per sample compared to one MGIT culture. Delays between sample collection and culture processing are unlikely to explain the low culture detection yield because as part of the study procedures, samples were processed within 24 h after collection.

Despite the small number assessed, we found no added value in pooling two sputum samples for culture and Xpert testing. Since our study was not designed to compare pooled testing vs individual testing of two sputum samples, but only used the pooled strategy to save cost and reduce laboratory workload, we were expecting a better detection yield when compared to single-specimen testing from patients unable to produce two samples. In an adult study, pooled testing strategy had a high level of agreement with individual testing, although it reduced costs, and had the potential to increase the affordability of Xpert MTB/RIF [33]. In another study, there was a linear relationship between the pooling ratio and the Xpert MTB cycle threshold (Ct) value, but the slope of increase was relatively small [34]. These findings suggest that there is not much difference between pooled samples and individual testing especially if the quality of sample is good. This approach requires further assessment in samples from children.

Table 5 Detection of Xpert MTB/RIF among pooled and non-pooled sputum samples

		Pooled samples, n (%)	One sample, n (%)	p value
Xpert, N = 350	Neg	273 (96.5)	61 (91.1)	0.156
	MTB	9 (3.2)	5 (7.6)	
	Invalid	1 (0.7)	1 (9.1)	
LJ, N = 355	Neg	265 (93.3)	63 (90)	0.726
	MTB	10 (3.9)	3 (4.2)	
	Contaminated	9 (2.8)	4 (5.5)	
	NTM	1	0	
MGIT, N = 355	Neg	247 (86.6)	55 (78.6)	0.195
	MTB	5 (2.1)	3 (4.2)	
	Contaminated	32 (11.3)	12 (16.7)	
	NTM	1 (0.4)	0	

LJ, Lowenstein Jensen medium; *MGIT*, *Mycobacterium* growth indicator tube; *MTB*, *Mycobacterium tuberculosis*; *NTM*, non-tuberculosis mycobacteria

Table 6 Performance of Xpert MTB/RIF in stool against culture or Xpert MTB/RIF in sputum

	Sputum culture or Xpert positive	Sputum culture and Xpert negative
Stool Xpert MTB/RIF		
MTB	5	1
Negative	4	54
Invalid	0	1
Sensitivity, n/N (%)	5/9 (55.6% 21.2–86.3)	
Specificity, n/N (%)	54/55 (98.2% 90.3–100)	
Positive predictive value, n/N (%)	5/6 (83.4% 35.9–99.6)	
Negative predictive value, n/N (%)	54/58 (93.1% 83.3–98.1)	

MTB, *Mycobacterium tuberculosis*

Among stool samples, the sensitivity was generally low for Xpert MTB/RIF but in the range of sensitivities (32 to 85%) reported by the few existing studies [8–12, 35–37], the specificity was high as previously reported. We observed four stool Xpert MTB/RIF-positive samples that were negative by stool culture similar to those reported in South Africa [37]. Previous studies have attributed this difference to stool sample collection occurring when the patient has already taken some anti-tuberculosis drugs that may kill the paucibacilli in the swallowed sputum [37]. In this study, stool samples were collected within 1 week of treatment and this could have affected the viability of *Mycobacterium tuberculosis*. One of the factors that contribute to the difference in performance of the Xpert MTB/RIF from stool is the use of different specimen processing methods by the studies to remove PCR inhibiting factors that are very common in stool [8–10, 12]. The need of such specimen processing phase is also a serious limitation for the use of the Xpert MTB/RIF from stool at a low level of health care facility. Some groups are developing stool specimen processing kits that could be easily used by a nurse [12].

This is particularly important knowing that recent studies have shown that Xpert MTB/RIF testing of stool and nasopharyngeal aspirate, which is also relatively easy to collect in young children, is performing as well as two induced sputa or two gastric aspirates [9, 38].

It is thus necessary to re-think and aggressively evaluate non-sputum-based methods that could provide alternative yet reliable diagnosis for childhood TB. Foundation for Innovative New Diagnostics has recently included a non-sputum-based biomarker test for all forms of tuberculosis, ideally suitable for use at levels below microscopy centres among the four high-priority TB diagnostic needs [39]. Evaluating the sensitivity of the Xpert MTB/RIF testing in stool samples using the Ultra cartridge recently released by Cepheid that has a level of detection close to the culture is needed [40, 41]. However, this method will still be limited by PCR inhibitors present in stool samples.

This study had some limitations including the fact that we did not randomise samples for pooling against individual testing. Secondly, the study did not extensively evaluate the performance of different tests on all stool samples from the entire cohort of children because the evaluation of Xpert MTB/RIF in stool was an exploratory objective at the time of the study design. Less than 50% of the cases were tested due to the difficulty to collect stools in outpatients. We feel there might have been a lack of supervision of mothers who were asked to collect stool. Since the stool was only collected among children started on TB treatment, it is highly possible that outpatient children left before producing the sample once they had received their treatment. However, other studies have shown good rate of stool collection between 61 and 98.2% but many included hospitalised children or did not distinguish between out and inpatients [8, 9, 11, 35]. The few microbiologically confirmed TB patients limited also the ability to evaluate the accuracy of Xpert MTB/RIF in stool. In addition, other samples like gastric aspirates and nasopharyngeal swabs were not included in the evaluation despite their potential to increase case detection rate in children.

Conclusion

This diagnostic study confirmed the good performance of Xpert MTB/RIF in sputum as compared to culture in children and highlights potential value of using Xpert MTB/RIF from stool sample. Further evaluation of Xpert MTB/RIF using more sensitive Ultra cartridge is necessary from stool combined with optimised specimen processing to improve MTB recovery. Finally, an additional study to compare the detection yield of Xpert MTB/RIF from pooled specimen (respiratory or others) is necessary to improve sensitivity in a cost-effective manner.

Compliance with ethical standards

All authors contributed sufficiently to the scientific work and therefore share collective responsibility and accountability for the results. Consent to submit this work has been received explicitly from all co-authors.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in this study were in accordance with the ethical standards of the institutional and national research committees and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Approvals were received from Mbarara University Research Ethics Committee, the Uganda National Council for Science and Technology and The Comité de Protection des Personnes of Ile de France XI, Saint-Germain, Laye, France.

Informed consent Informed consent was obtained from all individual participants included in this study. Particularly, written informed consent was given by the parent or legal guardian and assent by children of 7 years or older.

References

1. Marais BJ, Schaaf HS (2010) Childhood tuberculosis: an emerging and previously neglected problem. Infect Dis Clin North Am 24(3): 727–749

2. Jenkins HE (2016) Global burden of childhood tuberculosis. Pneumonia 8(1):24

3. WHO (2017). Global Tuberculosis Report. 2017(WHO/HTM/TB/ 2017.23)

4. WHO. (2014). Guidance for national tuberculosis programmes on the management of tuberculosis in children 2014

5. Marais BJ, Gie RP, Hesseling AC, Schaaf HS, Lombard C, Enarson DA, Beyers N (2006) A refined symptom-based approach to diagnose pulmonary tuberculosis in children. Pediatrics 118:e1350– e1359

6. Marais BJ, Gie RP, Schaaf HS, Hesseling AC, Obihara CC, Starke JJ, Enarson DA, Donald PR, Beyers N (2004) The natural history of childhood intra-thoracic tuberculosis: a critical review of literature from the prechemotherapy era. Int J Tuberc Lung Dis 8:392–402

7. Detjen AK, DiNardo AR, Leyden J, Steingart KR, Menzies D, Schiller I, Dendukuri N, Mandalakas AM (2015) Xpert MTB/RIF assay for the diagnosis of pulmonary tuberculosis in children: a systematic review and meta-analysis. Lancet Respir Med 3(6): 451–461

8. 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, Hesseling 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 2017

9. Marcy O, Ung V, Goyet S, Borand L, Msellati P, Tejiokem M, Nguyen Thi NL, Nacro B, Cheng S, Eyangoh S, Pham TH, Ouedraogo AS, Tarantola A, Godreuil S, Blanche S, Delacourt C (2016) Performance of Xpert MTB/RIF and alternative specimen collection methods for the diagnosis of tuberculosis in HIV-infected children. Clin Infect Dis 62:1161–1168

10. Nicol MP, Spiers K, Workman L, Isaacs W, Munro J, Black F, Zemanay W, Zar HJ (2013) Xpert MTB/RIF testing of stool samples for the diagnosis of pulmonary tuberculosis in children. Clinical Infectious Diseases 57(3):e18–e21

11. LaCourse SM, Pavlinac PB, Cranmer LM, Njuguna IN, Mugo C, Gatimu J, Stern J, Walson JL, Maleche-Obimbo E, Oyugi J, Wamalwa D, John-Stewart G (2018) Stool Xpert MTB/RIF and urine lipoarabinomannan for the diagnosis of tuberculosis in hospitalized HIV-infected children. AIDS 32(1):69–78

12. Banada PP, Naidoo U, Deshpande S, Karim F, Flynn JL, O'Malley M, Jones M, Nanassy O, Jeena P, Alland D (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)

13. Graham SM, Ahmed T, Amanullah F, Browning R, Cardenas V, Casenghi M, Cuevas LE, Gale M, Gie RP, Grzemska M, Handelsman E, Hatherill M, Hesseling AC, Jean-Philippe P, Kampmann B, Kabra SK, Lienhardt C, Lighter-Fisher J, Madhi S, Makhene M, Marais BJ, McNeeley DF, Menzies H, Mitchell C, Modi S, Mofenson L, Musoke P, Nachman S, Powell C, Rigaud M, Rouzier V, Starke JR, Swaminathan S, Wingfield C (2012) Evaluation of tuberculosis diagnostics in children: 1. Proposed clinical case definitions for classification of intrathoracic tuberculosis disease. Consensus from an expert panel. J Infect Dis 205(suppl_2): S199–S208

14. Rieder H, Van Deun A, Kam K M, Kim S J, Chonde T M, Trébucq A, Urbanczik R. (2007) Priorities for tuberculosis bacteriology services in low-income countries. http://www.theunion.org/what-we-do/publications/technical/priorities-for-tuberculosis-bacteriology-services-in-low-income-countries, 2007

15. Cepheid, Sunnyvale, California, United States. (2010). Product insert, 2010

16. Cuevas LE, Browning R, Bossuyt P, Casenghi M, Cotton MF, Cruz AT, Dodd LE, Drobniewski F, Gale M, Graham SM, Grzemska M, Heinrich N, Hesseling AC, Huebner R, Jean-Philippe P, Kabra SK, Kampmann B, Lewinsohn D, Li M, Lienhardt C, Mandalakas AM, Marais BJ, Menzies HJ, Montepiedra G, Mwansambo C, Oberhelman R, Palumbo P, Russek-Cohen E, Shapiro DE, Smith B, Soto-Castellares G, Starke JR, Swaminathan S, Wingfield C, Worrell C (2012) Evaluation of tuberculosis diagnostics in children: 2. Methodological issues for conducting and reporting research evaluations of tuberculosis diagnostics for intrathoracic tuberculosis in children. Consensus from an expert panel. J Infect Dis 2012(15):S209–S215

17. Nicol MP, Workman L, Isaacs W, Munro J, Black F, Eley B, Boehme CC, Zemanay W, Zar HJ (2011) Accuracy of the Xpert MTB/RIF test for the diagnosis of pulmonary tuberculosis in children admitted to hospital in Cape Town, South Africa: A descriptive study. Lancet Infect Dis 11:819–824

18. Zar HJ, Workman L, Isaacs W, Munro J, Black F, Eley B, Allen V, Boehme CC, Zemanay W, Nicol MP (2012) Rapid molecular diagnosis of pulmonary tuberculosis in children using nasopharyngeal specimens. Clin Infect Dis 55:1088–1095

19. Moore HA, Apolles P, de Villiers PJ, Zar HJ (2011) Sputum induction for microbiological diagnosis of childhood pulmonary tuberculosis in a community setting. Int J Tuberc Lung Dis 15:1185– 1190

20. Zar HJ, Hanslo D, Apolles P, Swingler G, Hussey G (2005) Induced sputum versus gastric lavage for microbiological confirmation of pulmonary tuberculosis in infants and young children: a prospective study. Lancet 365:130–134

21. Zar HJ, Tannenbaum E, Apolles P, Roux P, Hanslo D, Hussey G (2000) Sputum induction for the diagnosis of pulmonary tuberculosis in infants and young children in an urban setting in South Africa. Arch Dis Child 82:305–308

22. Zar HJ, Workman L, Isaacs W, Dheda K, Zemanay W, Nicol MP (2013) Rapid diagnosis of pulmonary tuberculosis in African children in a primary care setting by use of Xpert MTB/RIF on respiratory specimens: a prospective study. Lancet Glob Health 2013: 97–104

23. Bates M, O'Grady J, Maeurer M, Tembo J, Chilukutu L, Chabala C, Kasonde R, Mulota P, Mzyece J, Chomba M, Mukonda L, Mumba M, Kapata N, Rachow A, Clowes P, Hoelscher M, Mwaba P, Zumla A (2013) Assessment of the Xpert MTB/RIF assay for diagnosis of tuberculosis with gastric lavage aspirates in children in sub-Saharan Africa: a prospective descriptive study. Lancet Infect Dis 13:36–42

24. Zar HJ, Connell TG, Nicol M (2010) Diagnosis of pulmonary tuberculosis in children: new advances. Exp Rev Anti-Infect Ther 8: 277–288

25. Nicol MP, Zar HJ (2011) New specimens and laboratory diagnostics for child-hood pulmonary TB: progress and prospects. Paediatr Resp Rev 12:16–21

26. Jaganath D, Mupere E (2012) Childhood tuberculosis and malnutrition. J Infect Dis 206:1809–1815

27. Oliwa JN, Karumbi JM, Marais BJ, Madhi SA, Graham SM (2015) Tuberculosis and childhood pneumonia in tuberculosis endemic settings—common, cause or consequence? A review of the evidence. Lancet Resp Med 3:235–243

28. Acuña-Villaorduña C, Orikiriza P, Nyehangane D, White LF, Mwanga-Amumpaire J, Kim S, Bonnet M, Fennelly KP, Boum Y, Jones-López EC (2017) Effect of previous treatment and sputum quality on diagnostic accuracy of Xpert® MTB/RIF. Int J Tuberc Lung Dis 21(4):389–397

29. Kubica G, We D, Ml C, Middlebrook G (1963) Sputum digestion and decontamination with N-acetyl-L-cysteine-sodium hydroxide for culture of mycobacteria. Am Rev Respir Dis 87(5):775–779

30. Cadmus SI, Falodun OI, Fagade OE (2011) Methods of sputum decontamination with emphasis on local tuberculosis laboratories. Afr J Med Med Sci 40(1):5–14

31. Chien H, Yu MC, Wu MH, Lin TP, Luh KT (2000) Comparison of the BACTEC MGIT 960 with Löwenstein-Jensen medium for recovery of mycobacteria from clinical specimens. Int J Tubercul Lung Dis 4(9):866–870

32. Chihota GAD, Fielding K, Ndibongo B, van Zyl A, Muirhead D, Churchyard GJ (2010) Liquid vs. solid culture for tuberculosis: performance and cost in a resource-constrained setting. Int J tuberc Lung Dis 14(8):1024–1031

33. Abdurrahman MO, Lawson L, Oladimeji O, Blakiston M, Obasanya J, Dacombe R, Adams ER, Emenyonu N, Sahu S, Creswell J, Cuevas LE (2015) Testing pooled sputum with Xpert MTB/RIF for diagnosis of pulmonary tuberculosis to increase affordability in low-income countries. J Clin Microbiol 53(8):2502–2508

34. Ho J, Jelfs P, Nguyen PT, Sintchenko V, Fox GJ, Marks GB (2017) Pooling sputum samples to improve the feasibility of Xpert® MTB/RIF in systematic screening for tuberculosis. Int J Tuberc Lung Dis 21(5):503–508

35. Walters E, Gie RP, Hesseling 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(12):1316

36. Moussa HS, Bayoumi FS, Mohamed AM (2016) Gene Xpert for direct detection of mycobacterium tuberculosis in stool specimens from children with presumptive pulmonary tuberculosis. Ann Clin Lab Sci 46(2):198–203

37. Walters E, Demers AM, van der Zalm MM, Whitelaw A, Palmer M, Bosch C, Draper HR, Gie RP, Hesseling AC (2017) Stool culture for diagnosis of pulmonary tuberculosis in children. J Clin Microbiol 55(12):3355–3365

38. Song R (2015). Diagnostic yield of Xpert® MTB/RIF assay and mycobacterium tuberculosis culture on respiratory and non-respiratory specimens among Kenyan children. Abstract OA-440-05; 46th Union World Conference on Lung Health, Cape Town, 2015

39. Denkinger MC, Kik SV, Cirillo MD, Casenghi M, Shinnick T, Weyer K, Gilpin C, Boehme CC, Schito M, Kimerling M, Pai M (2015) Defining the needs for next generation assays for tuberculosis. journal of infectious diseases. 211:S29–S38

40. Dorman SE, Schumacher SG, Alland D, Nabeta P, Armstrong DT et al (2018) Xpert MTB/RIF Ultra for detection of Mycobacterium tuberculosis and rifampicin resistance: a prospective multicentre diagnostic accuracy study. Lancet Infect Dis 18(1):76–84

41. Nicol MP, Workman L, Prins M, Bateman L, Ghebrekristos Y, Mbhele S, Denkinger CM, Zar HJ. (2018). Accuracy of Xpert MTB/RIF ultra for the diagnosis of pulmonary tuberculosis in children. Pediatr Infect Dis J, 2018

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XPERT DIAGNOSIS OF CHILDHOOD TUBERCULOSIS FROM SPUTUM AND STOOL SAMPLES IN HIGH TB-HIV PREVALENT SETTINGS

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Abstract

Background: The Xpert MTB/RIF assay is a major advance for diagnosis of tuberculosis (TB) in high burden countries but is limited in children by their difficulty to produce sputum. We investigated TB in sputum and stool from children with the aim of improving pediatric TB diagnosis.

Methods: A prospective cohort of children with presumptive TB, provided 2 sputum or induced sputum at enrolment in Mbarara regional Referral Hospital, Uganda. Stool was collected from those started on TB treatment. All specimen were tested for Xpert MTB/RIF, MGIT, Lowenstein Jensen cultures and microscopy (except stool). We compared TB detection between age-categories and assessed performance of Xpert MTB/RIF in sputum and stool.

Results: Of the 392 children enrolled, 357 (91.1%) produced at least one sputum sample. Sputum, culture yield was 13/357 (3.6%): 3/109 (2.6%), 3/89 (3.2%), 3/101 (2.6%) and 4/44 (8.2%) among children of <2 years, 2-5 years, ≥5-10 years and >10 years, respectively (p=0.599). Xpert MTB/RIF yield was 14/350 (4.0%): 3/104 (2.9%), 4/92 (4.3%), 3/88 (2.9%) and 4/50 (.0%), respectively (p=0.283). Sensitivity and specificity of Xpert MTB/RIF in sputum against sputum-culture was 90.9% (95%CI 58.7 - 99.8), 99.1% (99.1- 99.8). In stool, it was 55.6% (21.2 - 86.3) and 98.2% (98.2 – 100) against Xpert MTB/RIF and culture in sputum.

Conclusion: Only a minority of children had microbiologically confirmed TB with a higher proportion in children above 10 years. Although sensitivity of Xpert MTB/RIF in stool was low, with good optimization, it might be a good alternative to sputum in children.

Chapter 3.2

Outcomes of children with presumptive tuberculosis in Mbarara, Rural Uganda

(Full manuscript publication attached at the end of this chapter)

3.2.1 Justification and objectives

Mortality among children with presumptive TB has been documented to be high in some settings. Among children not started on treatment, a systematic review reported a global case fatality rate of up to 21.9%, with a significantly higher proportion in the younger age group(163). The proportion of mortality is reported much lower (0.9%) among children who are started on treatment(163). Risk factors such as HIV and malnutrition completely change the trend even among children receiving treatment. Indeed, evidence shows that mortality rates in children undergoing TB treatment varies from 3.3% in Ethiopia to 6% in Thailand and the risk factors have been largely attributed to HIV and malnutrition (164,165). Since TB symptoms in children are non-specific, many deaths caused by the disease may be erroneously attributed to more common diseases(166). In the absence of a reference standard for diagnosis of childhood TB, the accuracy of treatment decisions cannot be adequately assessed and due to the difficulty to confirm the diagnosis of tuberculosis, missed diagnosis may potentially result in poor outcomes(167). In children, this is expected to be higher especially among those with other comorbidities, because of poor microbiological confirmation methods. Most cases of TB death are only discovered at autopsy. At the same time, mortality data is limited by lack or unreliability of postmortem reports mainly because of costs and lack of competent human resource capacity (168). Few studies have documented the outcomes of children with presumptive TB according to the TB treatment decision and the factors associated with poor outcomes.

Objectives

- To describe the mortality and the predictors of death among children with presumptive TB
- To assess the relation between TB treatment and mortality

3.2.2 Methods, results and conclusion

Methods

This study analysed data from the same population of children with presumptive TB as described in section 3.1 above. Baseline children's characteristics were compared across groups of children started on TB treatment confirmed with TB (at treatment start using Xpert or later with culture results), started on treatment without bacteriologic confirmation (empirical treatment) and not treated for TB. Survival analysis using Kaplan–Meier estimates was performed separately for the three groups and cox regression analysis was performed to identify risk factors of death. After initial assessment, all patients were censored at the date of death or at 3 months.

Results

Globally, the study reported mortality of 6.9% within three months with a higher proportion (10.7%) among those treated for TB compared to the non-treated children (4.5%). Among children with bacteriologically confirmed TB none of them died during treatment compared to 12.3% that were treated empirically. Moreover, nearly 10% of these children on empirical died within one month of starting treatment compared to non-treated ones. Severely malnourished children [adjusted hazard ratio (aHR), 9.86; 95% confidence interval (CI): 3.11–31.23] and those with chest radiographs suggestive of TB (aHR, 4.20; 95% CI: 0.93–19.01) were more likely to die. Children receiving empiric TB treatment had an increased risk of death (aHR, 2.37; 95% CI: 1.01–5.55) compared with children without treatment after adjustment for age, sex, HIV status and BCG vaccination.

Conclusion

The study concludes that there is high mortality in children receiving empirically TB treatment and highlights the difficulty in diagnosing childhood TB, The study also observes that the likelihood of starting empirical treatment is increased in children admitted in critical condition which can explain the high mortality in our study.

3.2.3 Involvement in this work

In this study I participated in protocol development and study implementation. As laboratory manager, I coordinated development of analytical plans, procedures and other document that were used to capture data from the laboratory. In addition, I supervised the laboratory activities to ensure that they were performed according to the good clinical and laboratory practice as well as laboratory quality management system. I also participated in data analysis and manuscript writing. Full publication is shown in corresponding pages.

Outcome of Children With Presumptive Tuberculosis in Mbarara, Rural Uganda

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Background: Mortality among children with presumptive tuberculosis (TB) empiric TB treatment can be high. We describe the predictors of death among children with presumptive TB, and the relation between treatment and mortality.

Methods: A prospective cohort of children with presumptive TB who underwent clinical assessment, chest radiograph, tuberculin skin test and sputum bacterial tests for TB was followed up for 3 months. TB diagnosis was based on mycobacterial, clinical and radiologic findings. Predictors of deaths were determined using cox regression model.

Results: Of 360 children included in the analysis, 31.4% were younger than 2 years; 31.6% were HIV infected and 11.3% were severely malnourished. One hundred forty (38.9%) were diagnosed with TB, 18 (13%) of whom were bacteriologically confirmed. At 3 months of follow up, 25 of 360 (6.9%) children had died: 15 of 140 (10.7%) were receiving TB treatment versus 10 of 220 (4.5%) were not receiving treatment ($P = 0.025$). Severely malnourished children [adjusted hazard ratio (aHR), 9.86; 95% confidence interval (CI): 3.11–31.23] and those with chest radiographs suggestive of TB (aHR, 4.20; 95% CI: 0.93–19.01) were more likely to die. Children receiving empiric TB treatment had an increased risk of death (aHR, 2.37; 95% CI: 1.01–5.55) compared with children without treatment after adjustment for age, sex, HIV status and Bacillus Calmette-Guérin (BCG) vaccination.

Conclusions: The high mortality in children receiving empirically TB treatment highlights the difficulty in diagnosing childhood TB, the increased likelihood of starting treatment in critically ill children and in children with chronic disease, and the possibility of misdiagnosis. It strengthens the need to invest further in early TB detection and diagnosing nonsevere illness.

Key Words: tuberculosis, treatment, children, mortality

(Pediatr Infect Dis J 2018;37:147–152)

In 2015, the World Health Organization (WHO) estimated that there were 1 million new cases of tuberculosis (TB) and 210,000 deaths because of TB among children.[1] However, only one-third of estimated cases (359,000) were notified to the WHO due to the lack of effective tools to diagnose TB in children, especially in resource limited settings. The pauci-bacillary nature of TB disease and challenges to obtain quality specimens in children reduces the yield of TB diagnostic tests, such as XpertMTB/RIF and culture, especially in young children.[2–4] This situation leads to the prescription of empiric TB treatment in most of the presumptive TB cases, without confirmation of disease.

Previous studies show that mortality of childhood TB in limited resource countries can vary between 6% and 11% with a trend of higher mortality among younger children.[5–8] In some sub-Saharan African countries, especially during the preantiretroviral therapy era, higher mortality was documented among smear-negative cases and HIV-infected children, highlighting the issue of potential misdiagnosis and coinfections.[8,9] In the absence of a reference standard for diagnosis of childhood TB, the accuracy of treatment decisions cannot be adequately assessed.[10] This study aimed to describe mortality and predictors of death in children with presumptive TB and to assess the relation between TB treatment and mortality.

METHODS

Study Population

The study was a prospective observational cohort of children 1 month to 14 years of age presenting to the pediatric outpatient and inpatient units of Mbarara regional referral hospital, Uganda, with presumptive TB. Presumptive TB was defined by the presence of at least one of the following symptoms or signs: reported fever for 7 days after exclusion of malaria; non - remittent cough or wheeze; night sweats, chest pain or unexplained apathy or reduced playfulness for the last 2 weeks; unexplained weight loss or documented failure to thrive over the past 3 months despite adequate nutrition and deworming; painless superficial lymph node mass(es); recent gibbus; or abdominal distention with ascites. Children with meningitis with lymphocytic predominance in cerebrospinal fluid or meningitis not responding to antibiotic treatment and those with a chest radiograph suggestive of TB were also eligible to be included in the study.[11] Children who completed a full course of TB treatment in the past 6 months, who were on treatment ≥ 3 days or on isoniazid prophylaxis and children living outside a 20 km radius around Mbarara city were excluded.

Procedures

At initial assessment, the parent/guardian was interviewed about the child's medical history, household TB contact history with a smear- or culture positive TB index case and child's symptoms followed by a physical examination. Bacillus Calmette-Guérin (BCG) vaccination was documented by the observation of a BCG scar or verbal information by the guardian. Tuberculin skin test was done using an intradermal injection of 2 tuberculin units (1 mL) of purified protein derivative 23 (Statens Serum Institute, Copenhagen, Denmark). Five and 10 mm cutoff were used to define positive results for HIV infected and noninfected children, respectively, regardless of nutritional status.[12] Posteroanterior and lateral chest radiograph digital images were obtained for all children, read and recorded by the doctor on-site using predetermined tick-sheets and classified according to a structured approach as follows: "normal," "abnormal suggestive of

TB" or "abnormal nonsuggestive of TB" (Supplemental Digital Content, http://links.lww.com/INF/C812). A suggestive radiograph was defined by the presence of hilar/paratracheal adenopathy with/without airway compression, airspace opacification not responding to antibiotics or with documented TB contact, lung cavities or miliary infiltrates. Children were offered HIV testing using the nationally approved testing algorithm with polymerase chain reaction (PCR) confirmation for positive children younger than 18 months.[13] Two sputum specimens were collected over 2 consecutive days using induced sputum for children unable to produce specimen, pooled and tested with XpertMTB/RIF assay (Cepheid, Sunnyvale, CA), two Lowenstein Jensen cultures and one Mycobacteria Growth Indicator Tube (MGIT) culture from each sputum sample at the Mbarara Epicentre Research Laboratory. Similar tests were performed on extra-pulmonary specimens collected according to the clinical presentation. Bacterial culture, cryptococcal antigen test, microscopy examination for cryptosporidium and cytology from lymph node aspiration or biopsy were irregularly available at the hospital laboratory. After initial assessment, children with any positive TB bacteriologic test, chest radiograph or clinical presentation that were suggestive of TB disease according to an experienced pediatrician were started on TB treatment using rifampicin (R), isoniazid (H), pyrazinamide (Z) FDC for 2 months followed by 4 months of isoniazid-rifampicin (HR) under drug observation by a family member.[14] Ethambutol (E) was added to the regimen during intensive phase in the presence of extensive disease (excluding TB meningitis), smear-positive results, HIV infection and/or suspicion of isoniazid drug resistance.[15] HIV infected children were referred to the hospital HIV clinic for appropriate treatment. Children started on TB treatment were followed monthly for 6 months with a last visit at month 12, and children who were not started on treatment had an additional study visit after 3 months.

Retrospective TB Case Classification

Digital images of chest radiograph were all read by an external radiologist who was blinded to the child's clinical presentation. The onsite reading and all patients' files were retrospectively reviewed by two independent senior pediatricians using the 2012 published consensus case definitions for childhood TB classifying children as confirmed, probable, possible, unlikely and not TB.[16] Possible cause of death was proposed after the review of serious adverse event reports and patients' file by two study clinicians including one senior pediatrician.

Sample Size and Data Analysis

Consecutive children with presumptive TB were recruited over 2 years. Data were entered using Voozanoo (Epiconcept, Paris, France) and were analyzed using Stata® 13 software (College Station, TX). Baseline children's characteristics (demographic, medical history, TB exposure, clinical, radiologic and bacteriologic) were summarized using frequencies and percentages for categorical variables, and medians and interquartile ranges (IQR) for continuous variables and were compared across groups of children started on TB treatment confirmed with TB (at treatment start using Xpert or later with culture results), started on treatment without bacteriologic confirmation (empirical treatment) and not treated for TB. Survival analysis using Kaplan–Meier estimates was performed separately for the three groups. After initial assessment, all patients were censored at the date of death or at 3 months. Predictors of death among baseline characteristics were explored using univariate and multivariate Cox regression model. Covariates associated with a P value < 0.4 in univariate analysis were included in the initial multivariate model; a manual backward stepwise approach was used to obtain the final multivariate model. Statistical significance ($P < 0.05$) was assessed with the likelihood-ratio test. In addition, the association between the decision to initiate treatment and mortality was assessed after adjusting on baseline characteristics that were not directly associated with the treatment decision (age, sex, HIV status and BCG vaccination).

The study was approved by the Mbarara University Research Ethics Committee, the Uganda National Council for Science and Technology and The Comité de Protection des Personnes of Ile de France XI, Saint-Germain, Laye, France. Written informed consent was given by the parent or legal guardian and assent by children of 7 years or older.

RESULTS

Of the 392 patients enrolled between April 2012 and January 2014, 144 (36.7%) were started on treatment. Majority (126 of 144; 87.5%) of them were not bacteriologically confirmed (Fig. 1). A total of 32 children (8.2%) were lost to follow-up and excluded from the analysis. This resulted in a total of 360 children: 18 treated with TB confirmation, 122 treated empirically and 220 not treated. Children treated empirically were more likely to be younger and malnourished as compared with the two other groups of children. Of the 18 children treated with TB confirmation, 13 (72.2%) had *Mycobacterium tuberculosis* (MTB) detected using Xpert (Table 1). A 2-year-old HIV-infected child with severe malnutrition and confirmed TB by Xpert died 7 days later at home without been started on TB treatment because parents left the ward before the child's discharge and the family could only be traced after few days. This child was analyzed in the group of nontreated cases. Of the 122 children on empirical treatment, 38 (31.1%) were classified as probable TB using the consensus case definitions for childhood TB. Of the 220 children not treated, 205 (93.2%) were classified as unlikely or not TB (Table 2).

A total of 25 of 360 (6.9%) children died during the first 3 months of follow-up, 15 of 140 (10.7%) among children treated for TB versus 10 of 220 (4.5%) among children not treated, $P = 0.025$. None of the 18 treated TB confirmed children died versus 15 of 122 (12.3%) for the group of children treated empirically (Fig. 2). The estimated cumulative mortality at 1 month of treatment in children started on empirical treatment was 9.8% (95% CI: 5.7 – 16.7), whereas in the group of children not treated it was 3.2% (95% CI: 1.5 – 5.6; Fig. 2). The median time to death was 11 days (IQR, 7–22) versus 20 days (IQR, 11–34) in the groups of children treated empirically and not treated, respectively. Most children who died were younger than 2 years (60.0%). Severe malnutrition (47.8%) and HIV infection (44.0%) were common among children with a fatal outcome (Table 3). Although nonsignificant, there was two-fold increased risk of death related to not having a BCG scar. Of the 121 children started on empirical treatment, 30 (24.5%) were HIV-positive, and of them 18 were on antiretroviral therapy (ART) at the time of TB diagnosis. There was a trend on higher mortality in the group of HIV-positive children not on ART (6 of 22; 26.3%) as compared with those already on ART (2 of 18; 11.1%) or who were HIV-negative (7 of 81; 8.6%), $P = 0.06$.

Using the consensus case definitions for childhood TB, of the 15 deaths from the group of children treated empirically for TB, two were classified as probable TB. However, in one of the two cases, death occurred three months after starting treatment and was due to chicken pox. For the second child, death occurred 2 days after starting TB treatment in a context of jaundice, hematemesis and fever. The other causes of death for children on empirical treatment classified as unlikely TB, not TB or unclassified were severe pneumonia with comorbidities for 12 children (HIV infection without ART and severe malnutrition in eight cases and only severe malnutrition in four cases) and suspicion of lymphoma in one HIV-positive child. Of the children nonstarted on TB treatment, the causes of death were TB (n = 1), acute cardiac failure
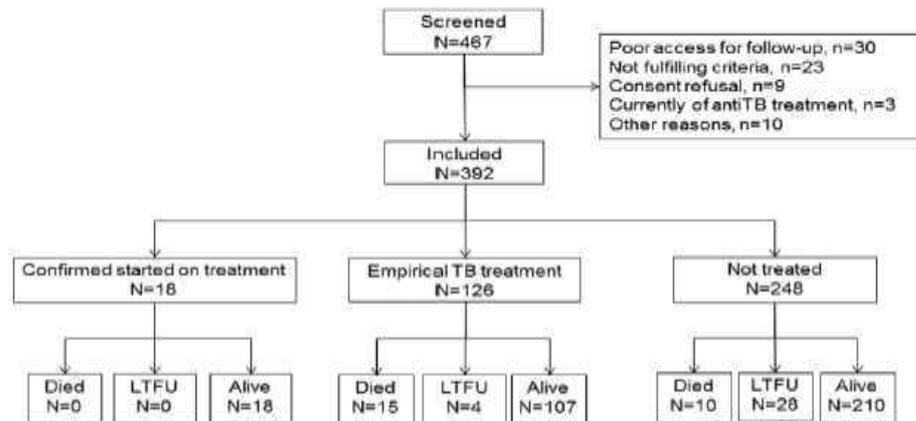
FIGURE 1. Study profile.

TABLE 1. Baseline Characteristics of Included Children According to Treatment Decision

Characteristics	All Cases (N = 360)	Treated Confirmed (n = 18)	Empirical Treatment (n = 122)	Not Treated (n = 220)	P
Sex, female	162 (45.0)	8 (44.4)	53 (43.4)	101 (45.9)	0.907
Age (y) category, n (%)					< 0.001*
< 2	113 (31.4)	4 (22.2)	59 (48.4)	50 (22.7)	
2–5	94 (26.1)	4 (22.2)	37 (30.3)	53 (24.1)	
5–10	102 (28.3)	4 (22.2)	17 (13.9)	81 (79.4)	
> 10	51 (14.2)	6 (33.3)	9 (7.4)	36 (70.6)	
HIV positive, n (%)	113/357 (31.6)	7/17 (41.2)	40/121 (33.0)	66/219 (30.1)	0.185
On ART, n (%)	68 (60.2)	3 (42.9)	18 (45.0)	47 (71.2)	0.018
Weight for height Z score, N	353	18	120	215	< 0.001*
Normal	211 (59.8)	7 (38.9)	50 (41.7)	154 (71.6)	
<−1 to −1.9 SD	72 (20.4)	7 (38.9)	27 (22.5)	38 (17.7)	
<−2 to −2.9 SD	30 (8.5)	1 (5.6)	17 (14.2)	12 (5.6)	
≤−3 SD	40 (11.3)	3 (16.7)	26 (21.7)	11 (5.1)	
BGC immunized, n (%)	312 (86.9)	14 (77.8)	102 (83.6)	196 (89.1)	0.091
TB contact history, n (%)	75 (20.8)	7 (38.9)	44 (36.4)	24 (10.9)	< 0.001
< 2 years old	23/113 (20.5)	3/4 (75.0)	17/58 (29.3)	3/50 (6.0)	< 0.001
TST positive, n (%)	95/351 (27.1)	8/17 (47.1)	59/117 (50.4)	28/217 (12.9)	< 0.001
Antibiotics in last 2 wk, n(%)	241 (66.9)	11 (61.1)	76 (62.3)	154 (70.0)	0.302
Clinical presentation, n (%)					
> 2 wk cough	330 (91.7)	17 (94.4)	103 (84.4)	210 (63.6)	0.002
≥ 7 d reported fever	136 (37.8)	8 (44.4)	57 (46.7)	71 (32.3)	0.026
> 2 wk night sweats	180 (50.0)	11 (61.1)	60 (49.2)	109 (49.8)	0.630
> 2 wk unexplained fatigue	125 (43.1)	9 (50.0)	60 (49.2)	56 (25.4)	< 0.001
Peripheral adenopathy	19 (5.3)	6 (33.3)	7 (5.7)	6 (2.7)	< 0.001
Chest radiograph, n (%)					
Consolidation	49 (13.6)	7 (43.7)	28 (23.5)	14 (6.5)	< 0.001
Cavity	4 (1.1)	1 (6.2)	2 (1.7)	1 (0.5)	0.086
Mediastinal adenopathy	68 (18.9)	7 (43.7)	54 (45.4)	7 (3.2)	< 0.001
Broncho-pneumonic patterns	147 (40.8)	12 (75.0)	70 (58.2)	65 (30.1)	< 0.001
Suggestive of TB	130 (36.1)	12 (75.0)	97 (81.5)	21 (9.7)	< 0.001
TB laboratory					
Sputum-positive culture	15 (4.2)	13/17 (76.5)	1/108 (0.9)†	1/205 (0.5)	
Xpert MTB detected	14 (3.9)	13/15 (86.7)	0/104	1/202 (0.5)	

SD indicates standard deviation; TST, tuberculin skin test.
*Pearson chi square.
†Non-TB mycobacteria.

with valvular heart disease (n = 2), severe malnutrition with nephrotic syndrome (n = 1), empyema due to staphylococcal pneumonia (n = 1), pyogenic pneumothorax without bacterial identification (n = 1); hypovolemic shock due to severe diarrhea and malnutrition (n = 1), suspicion of central neurologic opportunistic infection in HIV-infected children failing ART (n = 2) and bacteriologically unconfirmed severe pneumonia in a HIV infected child (n = 1). Among children started on empirical TB treatment, the case fatality of children classified as probable or possible TB was 3.9% (3 of 76) versus 24.3% (9 of 37) for children classified as unlikely TB or not TB, P = 0.001 (Table 2).

TABLE 2. Retrospective Case Review Using Consensus Case Definition for Children Started on Empirical TB Treatment and Children Not Started on TB Treatment

Review	Empirical Treatment	No Treatment
All cases, n (%)	N = 122	N = 220
Classified	113 (92.6)	213 (96.8)
Confirmed TB	0	1 (0.5)*
Probable TB	38 (33.6)	1 (0.5)
Possible TB	38 (33.6)	6 (2.8)
Unlikely TB	17 (15.0)	37 (17.4)
Not TB	20 (17.7)	168 (78.9)
Deaths, n (%)	N = 15	N = 10
Classified	12	9
Confirmed TB	0	1*
Probable TB	2	0
Possible TB	1	0
Unlikely TB	2	1
Not TB	7	7

Confirmed TB: 1 sign or symptom of TB + microbiologic confirmation. Probable TB: 1 sign or symptom of TB + chest radiograph suggestive of TB + positive clinical response to TB treatment, or TB contact history or positive tuberculin skin test. Possible TB: 1 sign or symptom of TB + positive clinical response to TB treatment or TB contact history or positive tuberculin skin test or chest radiograph suggestive of TB. Unlikely TB: 1 sign or symptom of TB but not fitting the above definitions and no alternative diagnosis established. Not TB: fitting the definition of unlikely TB but with an established alternative diagnosis.

*Child with XpertMTB positive result who died without been started on treatment.
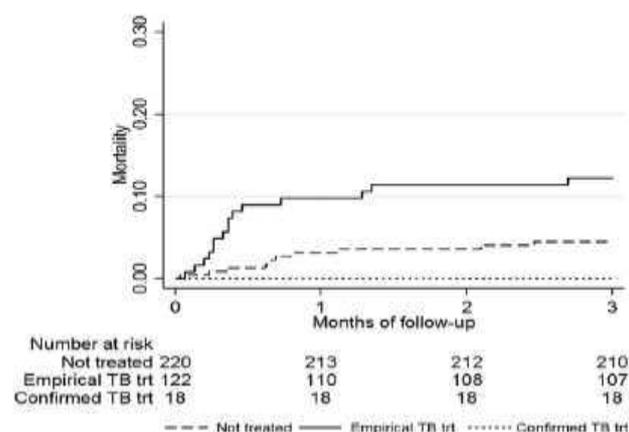
FIGURE 2. Three months survival curves for children treated TB confirmed; treated empirically and not treated children.

Patients' baseline characteristics independently associated with mortality were severe malnutrition (aHR, 9.86; 95% CI: 3.11–31.23) and chest radiograph suggestive of TB (aHR, 4.20; 95% CI: 0.93–19.01; Table 3). In univariate analysis, patients who were admitted after receiving a nonspecific antibiotic course within the past 2 weeks were more likely to die than children who did not receive antibiotics (HR, 3.5; 95% CI: 1.12–12.53). Although not significant, there was a trend on higher mortality among HIV-positive children not on ART as compared with HIV-negative children. After excluding baseline patients' characteristics that could be included in the decision to start or not a TB treatment at initial assessment (chest radiograph findings, TB contact history and weight for height score), being started on

empirical TB treatment was independently associated with death (aHR, 2.37; 95% CI: 1.01–5.55) compared with no treatment after adjustment for sex [female aHR, 0.49 (0.20–1.18)] and age [2–5 years aHR, 0.34 (0.11–1.04), 5–10 years aHR, 0.42 (0.13–1.33) and > 10 years aHR, 0.39 (0.88–1.78)].

DISCUSSION

In this cohort, we report more than two-fold increase of deaths among children started on TB treatment compared with those not treated (10.7% versus 4.5%), and among treated children, all death occurred among those started on empiric treatment. The mortality of children treated for TB is similar to what has been reported in previous studies.[5–9] Retrospective cohort studies have shown a trend of increased mortality in smear-negative children as compared with smear-positive ones.[5,6,9] Using more sensitive tests than microscopy (Xpert and culture), our study confirms this trend with no death among children treated with TB confirmation versus 12.3% among children treated empirically. While previous studies have shown an increased mortality among HIV-TB coinfected children, our study was not powered to evaluate the effect of HIV.[6] However, we noticed a trend of higher mortality in the group of HIV-positive children who were not on ART at the time of TB diagnosis. This highlights the needs of systematic HIV screening during pregnancy and access to early ART for infected children. The trend of lower mortality among children without BCG scar is consistent with previous studies reporting nonspecific effects of BCG vaccination beyond the protecting effect against TB, that could be explained by the epigenetic modulations in monocytes and increased responsiveness against unrelated stimuli.[17,18] Comparative mortality data for the group of nontreated children admitted for presumptive TB are very scarce. However, the mortality (4.5%) was close to what has been recently reported for admitted children including postdischarge mortality from high HIV burden countries.[19,20] The low proportion of non-TB treated cases retrospectively classified as confirmed or probable TB (1%) seems to indicate that the risk of missed diagnosis of TB was low in this cohort.

The increased mortality in the group of children receiving empirical treatment most likely reflects the increased likelihood of initiating an empiric TB treatment in children with either critical conditions, comorbidities or younger age and TB exposure.[14,21] Indeed, there were significantly more malnourished and younger children in this group compared with the two other groups. Both factors are known to be associated with an increased risk of death.[14,20,22] In our cohort, majority of deaths (12 of 15) from the group of TB-treated children occurred in severely malnourished children presenting with severe pneumonia. However, although malnutrition is known to increase the pneumonia-related mortality and children with severe malnutrition treated for pneumonia might have TB, the role of TB remains still unclear mainly due to the difficulty of TB diagnosis in this population.[23–26]

The other issue raised from the high mortality among children treated empirically is the risk of misdiagnosis of other infection or disease, especially in children with comorbidities. The proportion of potential misdiagnosis and its impact on mortality is very difficult to estimate in settings with limited diagnostic capacity and without autopsy report. Only 2 of 3 children started on empirical TB treatment were retrospectively classified as probable or possible TB (67.3%). This might indicate a nonnegligible proportion of children who did not require TB treatment.

The study had several limitations: (1) the main limitation was the absence of autopsy reports that prevent knowing how much TB contributed to death; (2) the lack of diagnostic tests for other infections such as blood culture and PCR for respiratory virus did not allow to further investigate the differential

TABLE 3.   Predictors of Deaths

Risk factors	N	Death, n(%)	HR	95% CI	aHR	95% CI
Age category (y)						
< 2	113	15 (13.3)	1		1	
2–5	94	4 (4.3)	0.30	0.10–0.91	0.49	0.15–1.54
5–10	102	4 (3.9)	0.27	0.09–0.82	0.86	0.26–2.85
> 10	51	2 (3.9)	0.28	0.06–1.21	0.62	0.13–2.92
Child sex						
Male	198	18 (9.1)	1		1	
Female	162	7 (4.3)	0.47	0.20–1.12	0.42	0.16–1.10
Child HIV status						
Negative	243	14 (5.8)	1			
Positive on ART	68	5 (7.3)	1.26	0.45–3.49		
Positive not on ART	45	6 (13.3)	2.46	0.94–6.39		
Contact history*						
No	284	24 (8.4)	1			
Yes	75	1 (1.33)	0.15	0.02–1.13		
BCG*						
No BCG scar	11	2 (18.2)	1			
BCG scar	312	22 (7.0)	0.35	0.08–1.49		
Antibiotic trial						
No	119	3 (2.5)	1			
Yes	241	22 (9.1)	3.5	1.12–12.53		
Weight for height Z score*						
Normal	211	5 (2.4)	1		1	
< –1 to –1.9 SD	72	6 (8.3)	3.62	1.10–11.86	2.94	0.86–9.99
< –2 to –2.9 SD	30	1 (3.3)	1.44	0.17–12.31	1.07	0.12–9.63
≤ –3 SD	40	11 (27.5)	13.95	4.84–40.18	9.86	3.11–31.23
Chest radiograph*						
Normal	120	2 (1.7)	1		1	
TB suggestive	130	16 (12.3)	7.79	1.79–33.88	4.20	0.93–19.01
Abnormal non-TB suggestive	101	6 (5.9)	3.60	0.73–17.82	3.29	0.66–16.51
Bacteriologically confirmed						
No	341	24 (7.0)	1			
Yes	19**	1 (5.3)	0.75	0.10–5.57		
Treatment decision						
No treatment	220	10 (4.0)	1		NA	NA
Empirical treatment	122	15 (12.3)	2.85	1.28–6.34	NA	NA
TB confirmed treatment	18	0	NA		NA	NA

SD indicates standard deviation; NA, not applicable; HR, hazard ratio.

*Missing values excluding from the multivariate analysis.

**Including the child with Xpert confirmed TB who died without been started on treatment.

diagnosis of TB; (3) with only one follow-up visit for nontreated children, we could not keep lost-to follow-up cases in the time-dependent analysis and we had to exclude them resulting in the loss of power and potential bias if some of the lost-to follow-up were dead; (4) we applied the standard case definitions of intrathoracic childhood TB for the review of the diagnostic accuracy for unconfirmed TB in a prognosis study, which was not the purpose of the development of these standard definitions. They were proposed for diagnostic studies to reduce intrastudy variability and their limitation for the diagnostic accuracy of unconfirmed TB is well recognized.[27]

The goal to reach zero death from TB in children spearheaded by the WHO includes taking every opportunity for intervention to improve diagnosis and treatment, especially among those presenting with severe clinical conditions or chronic disease.[28,29] This includes the use of empirical TB treatment for such group of children to prevent death from undiagnosed TB. However, the high mortality of children receiving empirical TB treatment strengthens the need to invest further in robust TB diagnosis, particularly in more sensitive, nonsputum based, point of care tests to identify true TB cases that can be cured.[16] TB detection approach may need to be adapted in the light of recent studies showing high proportion of undiagnosed TB in young children admitted with acute severe pneumonia and in children with severe malnutrition aiming for more rapid and systematic detection of TB in these groups.[24,25]

However, although better diagnostics are essential, it is important to consider and reduce non-TB cause of chronic diseases. In the context of lethal viruses and bacteria causing respiratory tract infections, there is also an urgent need to further invest in diagnosing non-TB severe illnesses that mimic TB to avert this high mortality in children empirically started on TB treatment.[30]

REFERENCES

1. World Health Organization. WHO | Global tuberculosis report 2016; 2016. Available at: http://www.who.int/tb/publications/global_report/en/. Assessed May 16, 2017.

2. Detjen AK, DiNardo AR, Leyden J, et al. Xpert MTB/RIF assay for the diagnosis of pulmonary tuberculosis in children: a systematic review and meta-analysis. *Lancet Respir Med*. 2015;3:451–461.

3. Dodd PJ, Gardiner E, Coghlan R, et al. Burden of childhood tuberculosis in 22 high-burden countries: a mathematical modelling study. *Lancet Glob Health*. 2014;2:e453–e459.

4. Perez-Velez CM, Marais BJ. Tuberculosis in children. *N Engl J Med*. 2012;367:348–361.

5. Muñoz-Sellart M, Yassin MA, Tumato M, et al. Treatment outcome in children with tuberculosis in southern Ethiopia. *Scand J Infect Dis*. 2009;41:450–455.

6. Russell GK, Merle CS, Cooke GS, et al. Towards the WHO target of zero childhood tuberculosis deaths: an analysis of mortality in 13 locations in Africa and Asia. *Int J Tuberc Lung Dis*. 2013;17:1518–1523.

7. Flick RJ, Kim MH, Simon K, et al. Burden of disease and risk factors for death among children treated for tuberculosis in Malawi. *Int J Tuberc Lung Dis*. 2016;20:1046–1054.

8. Drobac PC, Shin SS, Huamani P, et al. Risk factors for in-hospital mortality among children with tuberculosis: the 25-year experience in Peru. *Pediatrics*. 2012;130:e373–e379.

9. Harries AD, Hargreaves NJ, Graham SM, et al. Childhood tuberculosis in Malawi: nationwide case-finding and treatment outcomes. *The International Journal of Tuberculosis and Lung Disease : The Official Journal of the International Union Against Tuberculosis and Lung Disease.* 2002;6(5):424–431. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12019918. Accessed October 12, 2016.

10. Graham SM. Research into tuberculosis diagnosis in children. *Lancet Infect Dis*. 2010;10:581–582.

11. WHO | Guidance for national tuberculosis programmes on the management of tuberculosis in children; 2006. Available at: http://www.who.int/tb/publications/childtb_guidelines/en/. Accessed January 28, 2016.

12. CDC | TB | Fact Sheets - Tuberculin Skin Testing for TB. Available at: http://www.cdc.gov/tb/publications/factsheets/testing/skintesting.htm. Accessed March 10, 2016.

13. MOH. National antiretroviral treatment guidelines for adults, adolescents, and children; 2009:1–80. Available at: http://www.aidstar-one.com/sites/default/files/treatment/national_treatment_guidelines/Uganda_guidelines_2009.pdf. Accessed January 31, 2017.

14. World Health Organization. WHO | Guidance for national tuberculosis programmes on the management of tuberculosis in children; 2014. Available at: http://www.who.int/tb/publications/childtb_guidelines/en/. Assessed May 16, 2017.

15. Marais BJ, Schaaf HS, Donald PR. Management of tuberculosis in children and new treatment options. *Infect Disord Drug Targets*. 2011;11(2):144–56. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21406050. Accessed October 17, 2016.

16. Graham SM, Ahmed T, Amanullah F, et al. Evaluation of tuberculosis diagnostics in children: 1. Proposed clinical case definitions for classification of intrathoracic tuberculosis disease. Consensus from an expert panel. *J Infect Dis*. 2012;205 Suppl:S199–S208.

17. Benn CS, Fisker AB, Whittle HC, et al. Revaccination with live attenuated vaccines confer additional beneficial nonspecific effects on overall survival: a review. *EBioMedicine*. 2016;10:312–317.

18. Storgaard L, Rodrigues A, Martins C, et al. Development of BCG scar and subsequent morbidity and mortality in rural Guinea-Bissau. *Clin Infect Dis*. 2015;61:950–959.

19. Chhibber AV, Hill PC, Jafali J, et al. Child mortality after discharge from a health facility following suspected pneumonia, meningitis or septicaemia in rural Gambia: a cohort study. *PLoS One*. 2015;10:e0137095.

20. Tette EMA, Neizer M, Nyarko MY, et al. Changing patterns of disease and mortality at the Children's Hospital, Accra: are infections rising? *PloS one*. 2016;11(4):e0150387.

21. Nahid P, Dorman SE, Alipanah N, et al. Executive summary: Official American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America Clinical Practice Guidelines: treatment of drug-susceptible tuberculosis. *Clin Infect Dis*. 2016;63:853–867.

22. Jones KDJ, Berkley JA. Severe acute malnutrition and infection. *Paediatr Int Child Health*. 2014;34(Suppl 1):S1–S29.

23. Sonego M, Pellegrin MC, Becker G, et al. Risk factors for mortality from acute lower respiratory infections (ALRI) in children under five years of age in low and middle-income countries: a systematic review and meta-analysis of observational studies. *PloS one*. 2015;10(1):e0116380.

24. Oliwa JN, Karumbi JM, Marais BJ, et al. Tuberculosis as a cause or comorbidity of childhood pneumonia in tuberculosis-endemic areas: a systematic review. *Lancet Respir Med*. 2015; 3: 235–243.

25. Chisti MJ, Graham SM, Duke T, et al. A prospective study of the prevalence of tuberculosis and bacteraemia in Bangladeshi children with severe malnutrition and pneumonia including an evaluation of Xpert MTB/RIF assay. *PloS one*. 2014;9(4):e93776.

26. Chisti MJ, Tebruegge M, La Vincente S, et al. Pneumonia in severely malnourished children in developing countries—mortality risk, aetiology and validity of WHO clinical signs: a systematic review. *Trop Med Int Health*. 2009;14:1173–1189.

27. Graham SM, Cuevas LE, Jean-Philippe P, et al. Clinical case definitions for classification of intrathoracic tuberculosis in children: an update. *Clin Infect Dis*. 2015;61(Suppl 3):S179–S187.

28. Sandgren A, Cuevas LE, Dara M, et al. Childhood tuberculosis: progress requires an advocacy strategy now. *Eur Respir J*. 2012;40:294–297.

29. World Health Organization. Roadmap for childhood tuberculosis: towards zero deaths; 2013. Available at: http://apps.who.int/iris/bitstream/10665/89506/1/9789241506137_eng.pdf. Assessed May 16, 2017.

30. Zumla A, Al-Tawfiq JA, Enne VI, et al. Rapid point of care diagnostic tests for viral and bacterial respiratory tract infections—needs, advances, and future prospects. *Lancet Infect Dis*. 2014;14:1123–1135.

P a g e | **90**

Chapter 3.3

Stool Xpert and urine LAM for diagnosis of tuberculosis in children

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Manuscript writing ongoing

The full write-up of this work has been included in the pages below

Presentations related to this chapter

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Orikiriza P, Nampijja D, Nyehangane D, Ssekyanzi B, Byamukama O, Kemigisha E, Mwanga-Amumpaire J, Bonnet M. Accuracy of two non-sputum based tuberculosis diagnostic tests (Xpert MTB/RIF in stool and urine LAM) in children at increased risk of severe or disseminated tuberculosis. 49th Union World Conference on Lung Health; Hague, Netherlands. 26th October 2018: Poster Presentation.

3.3.1 Background

Tuberculosis diagnosis in children is still a major challenge partly because of absence of an effective diagnostic tool, which has been a major barrier in the identification of TB affected children especially in high burden and resource-limited countries. There are a number of testing methods but most of them rely on use of sputum that is not always available in young children below 2 years and particularly those with comorbidities such as severe malnutrition and HIV. We recently reported high mortality in children with presumptive TB that were started on empirical treatment. The likelihood of starting empirical treatment is increased in children admitted in critical condition which can explain the high mortality in our study. Some of these children might have disseminated TB, which could explain the negative results of respiratory samples and others might have other diagnoses. These results highlighted the need of better diagnostic test for severe forms of TB, in particular disseminated TB and to focus the evaluation of new tests in children at high risk of presenting disseminated TB. This group includes very young children (< 2 years) and children with immunosuppression either due to HIV infection or severe malnutrition and this guided the choice of the study population of this study. These children even more likely to be unable to produce sputum and even if alternative respiratory sample collection can be available, despite having disseminated TB they may have no or few bacilli in their respiratory tract resulting in negative test results from respiratory samples. Therefore, non-sputum based tests are crucial to diagnose TB in this particular population.

Unfortunately, very few options are available so far. Urine LAM is one test option that can be used on fresh urine sample. The test is recommended for use in HIV adults with CD4 below $100/mm^3$ with moderate sensitivity and high specificity, and has shown to be a good predictor of the risk of death in this population(169). At the time of the study development, there was limited data on the use of this test in children more so in those at high risk of disseminated TB. A study in South Africa reported sub-optimal accuracy against the reference standard with sensitivity and specificity as low as 48.3% (37.6-59.2) and 60.8% (56.1-65.3) (100). The study attributed the poor specificity to potentially contamination from the skin or fecal matter despite using urine bags in collection.

At the same time, microbiological testing on stool specimen has been shown to be able to detect children with TB disease. Xpert on stool is one test that would provide fast results using a non-sputum sample that is easily available in children for diagnosis of pulmonary TB and could potentially have a role in the detection of subclinical TB and disseminated TB. The need to carry out further evaluation in very sick children that usually get misdiagnosed and the evaluation of non-sputum based tests were key to the implementation of this study.

3.3.2 Objectives

- To determine the accuracy of Xpert in stool samples against microbiologically confirmed TB and a composite reference according to uniform case definitions in children with presumptive TB admitted with severe illness or high risk of disseminated TB.
- To determine the accuracy of urine LAM in children against microbiologically confirmed TB and a composite reference according to uniform case definitions in children admitted with severe illness or high risk of disseminated TB.

3.3.3   Methods

3.3.3.1 Study population and design

This was a prospective diagnostic study conducted among children at increased risk of disseminated or severe TB at the pediatric and nutritional ward of the Mbarara Regional Referral Hospital. We enrolled children from 2 months and those below 14 years with HIV infection or severe malnutrition, and clinical suspicion of TB defined by the presence of at least 2 criteria: cough > 2 weeks, reported fever > 1week, severe malnutrition, reduced playfulness, TB contact history or contact with a person presenting chronic cough within the past 2 years. Children with severe pneumonia according to WHO criteria and one of the following criteria were also eligible: TB contact history or contact history with a person presenting chronic cough or no clinical improvement after 48 hours of large spectrum antibiotic course or significant peripheral lymphadenopathy. Finally, children with any sign suggestive of TB meningitis or disseminated/miliary TB were enrolled. After obtaining written informed consent from

parent/guardian and assent from children > 7 years old, baseline clinical, radiological and laboratory evaluations were performed at different visits (Figure 9).

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│        Screening of children at time of admission to the paediatric ward │
│                 Age, comorbidity, clinical presentation                  │
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                                    ▼
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│                      Baseline assessment (Day 1)                         │
│ Day 1                                                                    │
│    • Verification of eligibility criteria                                │
│    • Guardian/parent's consent and child's assent                        │
│    • Medical questionnaire and physical exam                             │
│    • HIV test, FBC, biochemical test, CXR                                │
│    • TST: injection                                                      │
│    • Xpert & MTB culture sputum/IS/GA/NPA                                │
│    • Urine LAM                                                           │
│    • Xpert and culture on stool                                          │
│    • +- diagnosis of other infection                                     │
│ Day 2                                                                    │
│    • Urine LAM                                                           │
│    • Xpert stool                                                         │
│    • +- diagnosis of other infection                                     │
│    • Sample collection for biothec                                       │
│ Day 3                                                                    │
│    • TST reading                                                         │
│    • TB treatment decision: yes, no, pending                             │
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│                   Follow-up (Day 7, weeks 2, 8, 24)                      │
│    • Physical exam                                                       │
│    • Day 7 and week 8: FBC, biochemical test                             │
│    • Week 2: CXR                                                         │
│    • Weeks 2 and 8: sample collection for biothec                        │
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│         Assessment of patients' files by 2 independent assessors         │
│                          TB classification                               │
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CXR: Chest x-ray, FBC: Full blood count, IS: Induced sputum, NPA: Nasopharyngeal aspirate, MTB: Mycobacterium tuberculosis, TST: Tuberculin skin test

Figure 10: Scheme of study design

After medical history information, clinical evaluation, a gastric aspirate and induced sputum or nasopharyngeal aspirate (NPA), plus two stool and urine samples were collected on 2 consecutive days. All samples were sent to the Epicentre TB laboratory for evaluation. Microscopy, Xpert and TB culture were performed on all respiratory samples and Xpert and culture on the stool sample. Determine TM Urine-LAM Ag test was used on the urine samples. Two blood samples were collected for TB culture in case of fever (T$\geq$38°C) and extra-pulmonary samples, when indicated, for Xpert and culture. In case of death, after obtaining consent from parent/guardian for autopsy, necropsy samples were also tested with Xpert and culture. In addition to TB tests, two blood cultures were collected for children with fever, two direct immunofluorescence microscopy for *Pneumocystis jirovecii* from IS or NPA in HIV infected (or exposed < 18months) children with acute hypoxic pneumonia and in children < 2 years old with an acute hypoxic pneumonia and bacterial culture from body fluid according to clinical presentation were also performed.

The study received approval from Mbarara University Research Ethics Committee (01/04-15) and the Uganda National Council for Science and Technology (HS 1814).

3.3.3.2 Study procedures

Sample collection;

IS/NPA was performed before GA after at least 4 hours of fasting. In brief, NPA was collected by inserting a graduated suction catheter through the nostril into the oropharynx to stimulate a cough reflex with secretions aspirated mechanically. For IS, there was inhalation of 5ml of salbutamol and 5ml of 5% hypertonic saline was nebulized for 20 minutes combined with nasopharyngeal suction in order to get a minimum of 2ml of sputum. For GA, a nasogastric tube was inserted first in the morning before feeding or after at least four hours of fasting. At least 2ml of gastric contents was obtained and transferred into a sterile container. If the aspirate was less than 2 ml, 5 ml of normal saline was inserted down the tube, left for 2-3 min, and then aspirated. In order to neutralize the acidic gastric contents, an equal volume of sodium bicarbonate solution was added to the specimen and later transported in a cool box to the laboratory for analysis.

Fresh urine samples (20-40ml) were collected in sterile urine containers using catheter, unless the children were old enough to produce urine by themselves. Later, we supplemented the urine collection method gentle cleaning using antibiotic soap to minimise contamination. Sixty µl of urine was collected for immediate testing with the urine-LAM test on site by the nurse. Remaining urine from the first sample was subjected to bacterial culture and that from a second sample was stored at -80°C ultra-freezer.

Two stool samples were collected on two consecutive days by a trained research nurse. At least a tea spoonful of stool was collected from baby's pamper with help of the guardian and transferred to the TB laboratory.

Blood was collected by a trained study nurse from the paediatric ward and mother/guardians were explained to collect stool.

Sample processing

Respiratory samples (sputum, IS, GA, and NPA) and biopsies or autopsies were decontaminated within the biosafety cabinet of the level three TB laboratory. Either induced sputum or NPA was collected depending on acceptability of the method in the different age groups. An equal volume of N-acetyl-L-cysteine and sodium hydroxide solution (1.25% final concentration) was added, vortexed and allowed to stand at room temperature. After 20 minutes, the mixture was neutralized with phosphate buffer and centrifuged for 20 minutes in a refrigerated centrifuge. The pellet was re-suspended in 1.5 ml of Phosphate Buffered Saline (PBS), ready for inoculation into culture media and xpert testing.

Approximately 0.5g of stool sample was homogenized with saline solution, vortexed in the screw capped container for 10 seconds and left to stand for 5 minutes. After settling, 5 ml of supernatant was picked and transferred into a 50 ml centrifuge tube. An equal volume of NaOH-NALC solution was added at 1.5% final concentration, vortexed lightly and left to stand for 20 minutes followed by neutralization as described for respiratory samples above. In order to reduce errors from Xpert due to inhibitors and contamination in culture, the stool decontamination step was repeated on the sediment.

Xpert MTB/RIF assay:

The Xpert assay was performed according to the manufacturer's instructions for sediments. The homogenized pellet from respiratory, biopsy, autopsy or stool samples were combined with Xpert reagent in a ratio of reagent-sample 2:1. In brief, 500 µl of sediment was mixed with 1.5 ml sample treatment buffer, shaken vigorously, and incubated for 15 min at room temperature before it was transferred to the Xpert MTB/RIF assay cartridge.

The DetermineTM Urine-LAM Ag test:

Urine LAM was performed on a fresh sample by a trained nurse within 30min of collection at the study site. The procedure was performed according to the manufacturer's guidelines. In brief, 60 µl of urine was applied to the sample cartridge and results read semi-quantitatively after 25 minutes and positive results were reported from 1+ to 4+. Cartridges without a control bar after the test were considered invalid. To assess the inter-reader reproducibility all consecutive urine LAM from children enrolled after January 2007 (protocol amendment) were read by two independent readers. However, for the main analyses, only the first reader's result was considered.

Mycobacterium culture:

For solid media, two drops of processed and re-suspended pellet were inoculated into each of the two Löwenstein Jensen (LJ) slants, and incubated for a maximum of 56 days. The results were reported qualitatively as positive or negative. For the positive culture, Ziehl Nelseen (ZN) test was done to check for acid fastness, and an MPT 64 (SD Bioline)-Rapid Diagnostic Test to confirm presence of MTB. The average number of colonies on all the LJ tubes per specimen were reported following the WHO grading scale (170). All results were shared with the study clinician to guide on patient management.

For liquid media, Mycobacteria Growth Indicator Tube (MGIT), (Bactec 960 Becton and Dickinson, New Jersey, USA) was prepared with PANTA (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin cocktail) and OADC (Oleic acid albumin dextrose and catalase supplement) to enhance growth and control contaminants as recommended by the

manufacturer. The MGIT media were then inoculated with 500ul of re-suspended pellet and incubated for 42 days before reporting a negative result. Positive cultures were tested with Ziehl Neelsen AFB microscopy and blood agar culture to rule out presence of contamination. Cultures with AFB were confirmed for *Mycobacterium tuberculosis* complex using MPT 64 (SD Bioline)-Rapid Diagnostic Test.

Pneumocystis jiroveci pneumonia testing:

Direct qualitative immunofluorescence test (Bio rad, Hercules, USA) was used to microscopically examine cystic forms of *Pneumocystis jirovecii* using monoclonal antibody immunofluorescent stain according to manufacturer's instructions from IS or NPA in case of children unable to tolerate IS on two consecutive days. Stained specimens were examined with UV microscope and the oocysts appeared as bright apple-green, evenly or unevenly labelled. As an internal quality control measure, the reading was done by 2 different lab tech and in case of discrepancy, a third reader was involved in defining the final result. Results were reported as negative or positive accordingly.

Blood culture:

Two blood samples were collected two hours apart and aseptically inoculated into aerobic Bactec blood culture bottles (Becton and Dickinson, New Jersey, USA) that were tested on the automated Bactec 9240 instrument. Upon growth of organisms, a signal was observed on the instrument. A Gram examination was performed and the bottle was sub-cultured onto appropriate media, depending on the type of bacteria seen. Colonies were identified using standard methods and Antibiotic resistance profiles of the pathogens identified determined using the disk diffusion method and, when appropriate, E-tests for determination of the minimum inhibitory concentrations. In case of TB culture, Bactec Myco/F Lytic – Mycobacteria bottles were used (Becton and Dickinson, New Jersey, USA) in the same 9240 instrument. Cultures were followed up until 42 days in case of negative results. Positive growth in the culture tube was followed by blood agar and ZN testing as described in the MGIT culture above.

HIV and CD4 testing:

For HIV test, pre- and post-test counselling were performed. A first Determine® HIV rapid test was performed and confirmed with a 2nd rapid test StatPak®. In case of discordance, a Unigold®

test was done following the national algorithm. Children <18 months with a positive test were confirmed using PCR at the laboratory of the Mulago Hospital in Kampala. The absolute CD4 cell count and percentage results were performed using the BD FACS Count™ system at the laboratory of the ISS clinic in the MNRH.

TST testing

The Mantoux method was performed following the guidelines issued by the American Academy of Paediatrics (Starke 1996; Centre for Disease Control and Prevention 2009). A positive TST was defined by an induration >5mm in HIV infected or HIV exposed children and in malnourished children. For the other children, the cut-off was 10 mm.

Chest X-ray:

Digitalized postero-anterior and lateral Chest X-ray was obtained for all children. A specific itemised chest X-ray evaluation sheet was used to report radiological findings. Each X-ray was read by the site clinician and an independent radiologist and a 3rd reader in case of discordant findings for the purpose of the case classification and the application of the standard case definition. At the end of the study, using an algorithm each case was retrospectively classified as confirmed, unconfirmed, and unlikely TB using the adapted Clinical Case Definitions for Classification of Intrathoracic Tuberculosis Disease from 2015(156). Cases that could not be classified by the algorithm due to incomplete data had their files reviewed by an independent TB pediatrician for final classification. Cases who did not fit criteria were kept as unclassified.

3.3.3.3 Statistical analysis

For sample size determination, based on the data reported by Nicol et al, (100) we assumed that 40% of children included in the study would be defined as confirmed or unconfirmed TB and that 60% of them might be urine LAM positive. Therefore, with such estimates if we enrolled 250 children, we would be able to estimate the proportion of true urine LAM positive result ("sensitivity") with a 10% precision. Assuming that the specificity of the urine LAM was not lower than 80%, we would be able to estimate the proportion of true negative urine LAM results ("specificity") with at least 10% precision.

Data were entered using Voozanoo® (Epiconcept, Paris, France) database, analysed using Stata® 13 software (College Station, Texas, USA). Patients and sample characteristics were reported by age category; overall, <2 years and ≥ 2years and summarized using percentages. Continuous variables were presented as median and interquartile range (IQR). The yield from all the samples was assessed using microscopy, Xpert and different culture methods. Accuracy of Urine LAM and stool Xpert test was compared to both microbiologically confirmed TB and a composite of confirmed and unconfirmed TB according to uniform case definitions based on intention to diagnose approach. Sensitivity, specificity, negative and positive predictive values were presented with 95% Confidence interval. A positive microbiological reference standard was defined by any MTB culture or Xpert result TB positive from any sample collected that is not stool but which could include autopsy sample and a negative microbiological reference standard by one negative culture or Xpert result from at least two different samples (2 respiratory or 1 respiratory and another sample) without any positive result.

In the intention to diagnose approach, we considered a test positive if any of the test result was positive and negative when none was positive.

Kappa analysis was performed to assess the inter-reader analysis of the urine LAM test based on Landis and Koch 1977(171). In summary, the results were scaled as <0; Poor agreement, 0.0-0.20; Slight agreement, 0.21-0.40; Fair agreement, 0.41-0.60; Moderate agreement, 0.61-0.80; Substantial agreement, 0.81-1.0; Almost perfect agreement.

3.3.4 Results

Patients' and sample characteristics

A total of 238 patients were enrolled between September 2015 and March 2018. Out of these, 234 patient data was available for final analysis. They were all inpatients. Figure 10

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                    ┌─────────────┐
                    │     238     │
                    │   enrolled  │
                    └──────┬──────┘
                           │          ┌──────────────┐
                           ├─────────▶│ 4 did not    │
                           │          │  provide     │
                           │          │  samples     │
                           │          └──────────────┘
                    ┌──────▼──────┐
                    │     234     │
                    │   analysed  │
                    └──────┬──────┘
        ┌──────────────────┴──────────────────┐
 ┌──────────────┐                      ┌──────────────────┐
 │12 Microbiol. │                      │222 microbiol.    │
 │ref. positive │                      │ref. Negative     │
 │(culture/Xpert)│                     │(culture/Xpert)   │
 └──────┬───────┘                      └────────┬─────────┘
    ┌───┴───┐                              ┌────┴────┐
 ┌─────┐ ┌─────┐                       ┌─────┐   ┌─────┐
 │Stool│ │Urine│                       │Stool│   │ LAM │
 └──┬──┘ └──┬──┘                       └──┬──┘   └──┬──┘
  ┌─┴─┐   ┌─┴─┐                        ┌──┴──┐   ┌──┴──┐
```

| 6 Xpert positive | 6 Xpert negative | 6 LAM positive | 6 LAM negative | 2 Xpert positive | 212 Xpert negative | 55 LAM positive | 157 LAM negative |
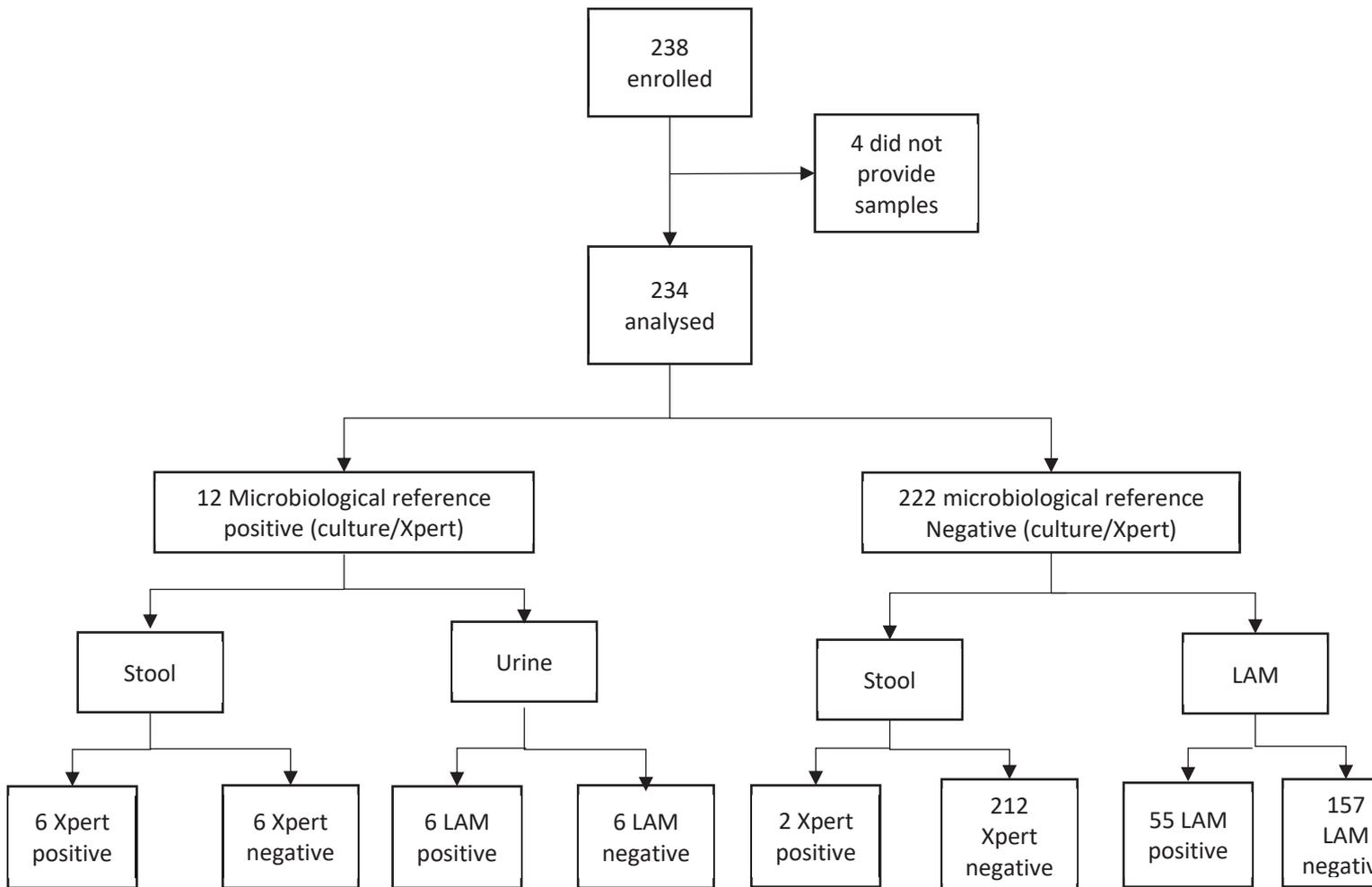
LAM: Urine LAM, Xpert: Genexpert testing, Culture: TB testing on LJ and MGIT

Figure 11: Schematic study profile

Overall, 48.3 % (113/234) children were females with median age of 16.5 months (10-36) as shown in Table 2. The overall HIV prevalence was high 31.6% (74/234), with higher rates among children ≥ 2 years. In addition, BCG vaccination was well covered at 96% (218/234). Largely, there was a

low history of contact with a bacteriologically confirmed TB case 3.4% (8/234). Majority of children had cough for more than two weeks; 82.5% (193/234) and a high proportion had been on antibiotic therapy; 87.2% (204/234). Severe malnutrition was high 58.5% (137/234) especially among children below 2 years, and 28.2% (66/234) of children had a clinical presentation of severe pneumonia.

Table 2: Patients' characteristics

Characteristics, n/N (%)	Overall N=234	<2 years, N=161	≥2 years, N=73	p
Sex, female	113(48.3)	76(47.2)	37(50.7)	0.622
Age(in months), median (IQR)	16.5(10-36)	13.0(8-17)	50.9(37-84)	
HIV positive	74(31.6)	43(26.7)	31(42.5)	0.016
CD4 cell count, median [IQR]	262.5[17-868]	587.0(69-1000)	162.0(13-840)	
BGC vaccination	218/227(96.0)	149(96.8)	69(94.5)	0.579
TB contact history				
With confirmed TB case	8 (3.4)	6(3.7)	2(2.7)	0.700
With non-confirmed TB case	10(4.3)	8(5.0)	2(2.7)	0.435
With a person presenting chronic cough*	15(6.4)	10(6.2)	5(6.8)	0.854
Received antibiotics before inclusion	204(87.2)	140(87.0)	64(87.7)	0.880
Clinical findings				
Cough > 2 weeks	193(82.5)	133(82.6)	60(82.2)	0.938
Reported fever for > 7 days	157(67.1)	106(65.8)	51(69.9)	0.544
Night sweats > 2 weeks	55(23.5)	36(22.4)	19(26.0)	0.540
Fatigue, weakness of reduced playfulness > 2 weeks	215(91.9)	145(90.1)	70(95.9)	0.131

Failure to gain weight > 3 months	103(44.0)	73(45.3)	30(41.1)	0.544
Nutritional status				
Weight for height <-2SD to -3SD	30(12.8)	22(13.7)	8(11.0)	0.566
Weight for height <-3SD	137(58.5)	103(64.0)	34(46.6)	0.012
MUAC < 115mm (<5years)	134(57.3)	120(74.5)	14(19.2)	<0.001
Bilateral oedema	39(16.7)	16(9.9)	23(31.5)	<0.001
Body temperature > 37.5°C	82(35.0)	55(34.2)	27(37.0)	0.675
Oxygen saturation <90%	14(6.0)	13(8.1)	1(1.4%)	0.045
Lymphadenopathy	56(23.9)	37(23.0)	19(26.0)	0.613
Positive TST	7(3.0)	5(3.1)	2(2.7)	0.879
Chest X-ray findings				
Normal	88(37.6)	63(39.1)	25(34.3)	0.475
TB suggestive	20/128(15.6)	14/90(15.6)	6/38(15.8)	0.973
Abnormal non-TB suggestive	108/128(84.4)	76/90(84.4)	32/38(84.2)	0.973

IQR: interquartile range

Overall, 97.4% (228/234) produced at least one respiratory related sample with no difference according to age category (Table 3). Children < 2 years were more likely to produce two samples than children ≥ 2 years, with the most frequent combination being GA+NPA. For stool, 99.6% (233/234) of children, produced at least one sample and 93.6% (219/234) patients gave two stool samples.

Table 3. Samples' characteristics per patient and age category

n (%)	Overall, N=234	<2 years, N=161	≥2 years, N=73	P value
Respiratory samples				
≥1 sample	228(97.4)	158(98.1)	70(95.9)	0.314
2 samples	225(96.2)	158(98.1)	67(91.8)	0.019
NPA+GA	195(83.3)	155(96.3)	40(54.8)	<0.001
Sputum/IS+GA	11(4.7)	1(0.6)	10(13.7)	<0.001
1 sample only	3(1.3)	0(0.0)	3(4.1)	0.010
Sputum	2(0.9)	0(0.0)	2(2.7)	
GA	1(0.4)	0(0.0)	1(1.4)	0.035
Stool samples				
≥1 sample	233(99.6)	161(100.0)	72(98.6)	0.137
2 samples	219(93.6)	155(96.3)	64(87.7)	0.013
1 sample only	14(6.0)	6(3.7%)	8(11.0)	0.031
Urine samples				
≥1 sample	231(98.7)	159(98.8)	72(98.6)	0.936
2 samples	219(93.6)	153(95.0)	66(90.4)	0.181
1 sample only	12(5.1)	6(3.7)	6(8.2)	0.149

NPA; Nasopharyngeal aspirate, GA: Gastric aspirate, IS: Induced sputum

Among all respiratory samples tested, the overall detection yields by microscopy, LJ culture, MGIT culture and Xpert were 2.2% (10/453), 2.6% (11/430), 2.8% (11/400) and 2.6% (12/452) respectively, after excluding contaminants, NTM results for culture and invalids results for Xpert (Table 4).

Table 4: Mycobacteriological results from all respiratory samples and per sample

Microscopy results, n(%)	All samples, N=453	Sputum, N=33	IS, N=12	GA, N=213	NPA, N=195
Negative	443(97.8)	33(100)	12(100)	208(97.7)	190(97.4)
Scanty	4(0.9)	0(0.0)	0(0.0)	1(0.5)	3(1.5)
1+	3(0.7)	0(0.0)	0(0.0)	2(0.9)	1(0.5)
2+	2(0.4)	0(0.0)	0(0.0)	2(0.9)	0(0.0)
3+	1(0.2)	0(0.0)	0(0.0)	0(0.0)	1(0.5)
LJ culture results, n(%)	All samples, N=453	Sputum, N=33	IS, N=13	GA, N=209	NPA, N=198
MTBc positive	11(2.4)	2(6.1)	0(0.0)	5(2.4)	4(2.0)
Negative	419(92.5)	31(93.9)	12(92.3)	189(90.4)	187(94.4)
Contaminated	23(5.1)	0(0.0)	1(7.7)	15(7.2)	7(3.5)
MGIT culture results, n(%)	All samples, N=453(%)	Sputum, N=33(%)	IS, N=13(%)	GA, N=209(%)	NPA, N=198(%)
Negative	389(85.9)	28(84.8)	11(84.6)	174(83.3)	176(88.9)
MTBc	11(2.4)	1(3.0)	0(0.0)	6(2.9)	4(2.0)
NTM	2(0.4)	0(0.0)	0(0.0)	2(1.0)	0(0.0)
Contaminated	51(11.3)	4(12.1)	2(15.4)	27(12.9)	18(9.1)
Xpert MTB/RIF assay, n(%)	All samples, N=453	Sputum, N=33	IS, N=12	GA, N=213	NPA, N=195
Negative	440 (97.1)	33(100)	12(100)	204(95.8)	191(97.9)
MTBc	12(2.6)	0(0.0)	0(0.0)	8(3.8)	4(2.1)

Invalid	1(0.2)	0(0.0)	0(0.0)	1(0.5)	0(0.0)
RIF Sensitive	12(100)	0(0.0)	0(0.0)	8(100)	4(100)

NPA; Nasopharyngeal aspirate, GA: Gastric aspirate, IS; Induced sputum, MTBc; Mycobacteria tuberculosis complex, RIF; Rifampicin, LJ; Lowenstein Jensen, NTM; Non tuberculous mycobacteria

Among all the MTB positive samples, NPA + stool contributed to a total of 75% (12/16). Among 24 children with autopsy samples tested for TB, 2 had MTB detected using Xpert assay.

At patient level, Xpert among patients with at least one sample yielded 3.4% (8/228) overall and 3.1% (5/158) and 4.1% (3/70) respectively among children below 2 years and those aged ≥2 years. The yield with GA was higher than with NPA; 3.8% (8/213) vs 2.1% (4/195).

For culture, among patients with at least one sample, the overall yield was 3.4% (8/228) while it was 3.1% (5/158) and 4.1% (3/70) respectively among children below 2 years and those aged ≥2 years. Furthermore, out of 56 children with severe pneumonia, 5.5% (3/56) were bacteriologically confirmed using Xpert.

Classification of cases using the uniformed case definitions

Overall, 5.1% (12/234) children had a confirmed diagnosis; 5% (8/161) and 5.5% (4/73) among those <2 years and ≥2 years respectively. Likewise, 28.6% (67/234) of children had unconfirmed TB; 23% (37/161) and 18.6% (30/161) among those <2 years and ≥2 years respectively. Largely, among unconfirmed TB, 1.3 % (3/234) were classified based on TST reaction and at least 1 TB suggestive sign while 27.4% (64/234) had a negative TST with at least one suggestive criteria. Also, 56.4% (132/234) had unlikely TB; 65.2% (105/161) and 37% (27/73) among those <2 years and ≥2 years respectively. Overall, 9.8% (23/234) were unclassifiable.

Performance of Xpert from stool

The overall sensitivity, specificity, negative and positive predictive values of Xpert on stool were; 50% (21.1-78.9), 99.1% (96.7-99.9), 75.0% (34.9-96.8) and 97.2% (94.1-99.0) against the microbiological reference standard, respectively (Table 5). The sensitivity remained the same

(50%) in the two age groups. The sensitivity was lower; 25% (19.4-99.4) among HIV-positive children compared to 62.5% (8.5-75.5) in non-infected ones.

Table 5: Diagnostic accuracy of Xpert in stool using a microbiological reference standard overall, per age category and co-morbidity

Any stool	SE n/N, % (95%CI)	SP n/N, % (95%CI)	PPV n/N, % (95%CI)	NPV n/N, % (95%CI)
Overall	6/12, 50 (21.1-78.9)	212/214, 99.1 (96.7-99.9)	6/8, 75.0 (34.9-96.8)	212/218, 97.2 (94.1-99.0)
< 2 years	4/8, 50 (15.7-84.3)	150/150, 100 (97.6-100)	4/4, 100 (39.8-100)	150/154, 97.4 (93.5-99.3)
≥ 2 years	2/4, 50 (6.8-93.2)	62/64, 96.9 (89.2-99.6)	2/4, 50 (6.8-93.2)	62/64, 96.9 (89.2-99.6)
HIV positive	1/3, 25 (19.4-99.4)	67/67, 100 (94.6-100)	1/1, 100(2.5-100)	67/70, 95.7 (88.0-99.1)
HIV negative	5/8, 62.5 (8.5-75.5)	140/142, 98.6 (95.0-99.8)	5/7, 71.4(29.0-96.3)	140/143, 97.9 (94.0-99.6)
Severe malnutrition	4/9, 44.4 (21.2-86.3)	171/173, 98.8 (95.9-99.9)	4/6, 66.7(22.3-95.7)	171/176, 97.2 (93.5-99.1)

Sensitivity, specificity, positive and negative predictive values of Xpert on stool were further assessed against composite reference cases overall, among the two age groups, HIV and nutritional status (Table 6). Overall, it was 10.1% (81.0-95.5), 100% (97.2-100), 100% (63.1-100) and 65.0% (58.0-71.6) respectively.

Table 6: Diagnostic accuracy of Xpert from stool against composite reference overall and per age category and co-morbidity

Any stool	SE n/N, % (95%CI)	SP n/N, % (95%CI)	PPV n/N, % (95%CI)	NPV n/N, % (95%CI)
Overall	8/71, 10.1 (81.0-95.5)	132/132, 100 (97.2-100)	8/8, 100(63.1-100)	132/203, 65 (58.0-71.6)
< 2 years	4/45, 8.9 (78.8-97.5)	105/105, 100(96.5-100)	4/4, 100 (39.8-100)	105/146, 71.9 (63.9-79.0)
≥ 2 years	4/34, 11.8 (72.5-96.7)	27/27, 100 (87.2-100)	4/4, 100 (39.8-100)	27/57, 47.4 (34.0-61.0)
HIV positive	1/20, 4.8 (76.2-99.9)	41/41, 100(91.4-100)	1/1, 100 (2.5-100)	41/61, 67.2(54.0-78.7)
HIV negative	7/58, 12.1 (76.7-95.0)	85/85, 100(95.8-100)	7/7, 100 (59.0-100)	85/136, 62.5 (53.8-70.6)
Severe malnutrition	4/9, 44.4 (21.2-86.3)	171/173, 98.8 (95.9-99.9)	4/6, 66.7 (22.3-95.7)	171/176, 97.2 (93.5-99.1)

Performance of urine LAM

For the urine LAM test, sensitivity, specificity, PPV and NPV were 50% (21.1-78.9), 74.1% (67.6-79.8), 9.8% (3.7-20.2) and 96.3% (92.2-98.8) against a microbiological reference standard respectively (Table 7). The sensitivity remained the same (50%) regardless of age group and HIV status or severe malnutrition.

Table 7: Diagnostic accuracy of urine LAM using a microbiological reference standard overall and per age category and co-morbidity

Any Urine	SE n/N, % (95%CI)	SP n/N, % (95%CI)	PPV n/N, % (95%CI)	NPV n/N, % (95%CI)
Overall	5/12, 50(21.1-78.9)	201/212, 74.1 (67.6-79.8)	5/16, 9.8 (3.7-20.2)	201/208, 96.3 (92.2-98.6)
< 2 years	4/8, 50 (15.7-84.3)	108/214, 73 (65.1-79.9)	4/44, 9.1 (2.5-21.7)	108/112, 96.4 (91.1-99.0)
≥ 2 years	2/4, 50 (6.8-93.2)	49/64, 76.6 (64.3-86.2)	2/17, 11.8 (1.5-36.4)	49/51, 96.1 (86.5-99.5)
HIV positive	2/4, 50 (6.8-93.2)	51/65, 78.5 (66.5-87.7)	2/16, 12.5 (1.6-38.3)	51/53, 96.2 (87.0-99.5)
HIV negative	4/8, 50 (15.7-84.3)	102/142, 71.8 (63.7-79.1)	4/44, 9.1 (2.5-21.7)	102/106, 96.2 (90.6-99.0)
Severe malnutrition	5/9, 55.6 (13.7-78.8)	121/170, 71.2 (63.7-77.9)	5/54, 9.3 (3.1-20.3)	121/125, 96.8 (92.0-99.1)

The accuracy of urine LAM test was assessed against composite reference cases overall and among the four categories. Overall sensitivity, specificity, positive and negative predictive values were 26% (62.8-83.4), 72.7% (64.3-80.1), 35.7% (23.4-49.6) and 62.7% (54.6-70.4) respectively. This did not change significantly among the different stratifications of age, HIV status and malnutrition (Table 8).

Table 8: Diagnostic accuracy of urine LAM against composite reference overall and per age category and co-morbidity

Any Urine	SE n/N, % (95%CI)	SP n/N, % (95%CI)	PPV n/N, % (95%CI)	NPV n/N, % (95%CI)
Overall	20/77, 26 (62.8-83.4)	96/132, 72.7 (64.3-80.1)	20/56, 35.7 (23.4-49.6)	96/153, 62.7 (54.6-70.4)
< 2 years	12/43, 27.9 (56.3-84.7)	78/105, 74.3 (64.8-82.3)	12/39, 30.8 (17.0-47.6)	8/17, 71.6 (62.1-79.8)
≥ 2 years	8/34, 23.5 (58.8-89.3)	18/27, 66.7 (46.0-83.5)	8/17, 47.1 (23.0-72.2)	18/44, 40.9 (26.3-56.8)
HIV positive	5/20, 25 (50.9-91.3)	31/41, 75.6 (59.7-87.6)	5/15, 33.3 (11.8-61.6)	31/46, 67.4 (52.0-80.5)
HIV negative	15/57, 26.3 (60.3-84.5)	60/85, 70.6 (59.7-80.0)	15/40, 37.5 (22.7-54.2)	60/102, 58.8 (48.6-68.5)
Severe malnutrition	16/54, 29.6 (56.4-82.0)	81/115, 70.4 (61.2-78.6)	16/50, 32.0 (19.5-46.7)	81/119, 68.1 (58.9-76.3)

In order to explore the high proportion of false positive LAM results, we assessed the inter-reader agreement. The overall positive-negative agreement by two readers was 249/252 (99.6%) and 28/33 (84.8%) tests respectively giving a kappa statistic of 0.888.

In addition, we explored if the false positive urine LAM results were more associated with bacterial urine contamination and the inter sample difference in Urine LAM grade (Table 9). Overall, 17 pathogens were isolated; 16 of which among false positive urine LAM test. Surprisingly, 28 tests had only one LAM test positive among the 55 samples. Also interestingly, there were 27 false positive samples with a weak band described as grade one.

Table 9: Urine bacterial results according to urine LAM results

n (%)	False positive LAM N=55	True positive LAM N=6
Grade 1 LAM results	27 (49.1)	0
Bacterial pathogen in the urines	16 (29.1)	1 (16.7)
Only one LAM positive out of two	28 (50.1)	0

3.3.5 Discussion and conclusion

In this population, HIV prevalence was generally high (31.6%) and 49.1% of children presented severe malnutrition; 68.8% were less than 2 years old, all of which are factors known to be associated with an increased risk of disseminated TB and severe form of TB once infected with MTB. More than a quarter of children had a clinical presentation of severe pneumonia, which is known to be associated with high risk of death.

Despite the high vulnerability of the children study population, the proportion of microbiologically confirmed TB was unexpectedly low (3.4%). It is quiet surprising that despite a wide scope of samples and high yield of respiratory sample collection (96.2% had two sample collected) and diagnostic tests used (Xpert and two different culture methods) and the fact that majority (82%) had cough of more than 2 weeks, the rate remains low. In our study, GA samples were neutralized with sodium bicarbonate; which has been shown to lower the culture yield in India(111). However, this effect is unlikely since Xpert equally missed the MTBc detection. Although we expected a high proportion of true TB cases to be missed by Xpert or culture testing of respiratory samples in children, due to the paucibacillary nature of childhood intrathoracic TB as already described, the proportion of children classified as unconfirmed TB using the standard case definition was low in our study (28.6%). It was also lower than 48% described in another South African study among admitted children with presumptive TB (172). Others have reported much higher unconfirmed TB cases (79.4%) among HIV infected populations across eight sites of Asia and Africa considered as high TB prevalence countries(120). However, the rate was slightly higher than that (17.6%) described by a group in South America within a similar study population (173). Variation of TB prevalence across countries and level of exposure can probably explain these differences. In our study only 3.4% of children had contact history with a confirmed case. Therefore, this questions the hypothesis of high TB prevalence in the study population.

Despite few confirmed cases, stool Xpert detected half of confirmed TB patients with a very high specificity. The average sensitivity of Xpert in stool is 67% (52-79) and specificity of 99% (98 to 99%) according to a recent meta-analysis. Higher sensitivity is expected among HIV infected children (79% vs 60%), although this was not observed in our study(122). However due to the

variability of the methods used for specimen processing, of study population and lack of standardization of the methodology, the authors concluded that the generalizability of the evidence is limited. Our results were in the range of sensitivity (32-89%) and specificity (95-99%) of studies using similar stool processing method based on the PBS and centrifugation(160,161,174–176). It was also close to what has been reported by studies using flotation method with a Sheather solution with sensitivities of 62 and 57%, and better to what has been recently published with the stool processing kits based on a sedimentation method without centrifugation (25%)(120,162,177). Therefore, further evaluation of Xpert from stool using optimized specimen processing method and standard methodology approach is still needed.

For urine LAM, we found a sensitivity of 50% which is close to what was previously reported(100). However, as it was observed in the first South-African study, the specificity was generally poor at 74.1% indicating many false positives. Few available data in children also show poor specificity in relation to the bacteriological confirmation. In Indonesia, sensitivity and specificity were much lower; 33% and 60%(178), while it was 43%(10-82) and 91%(84-95) in hospitalized HIV infected children in Kenya (161) respectively. In the Kenyan study, sensitivity of urine LAM increased to 60% (15-95) with severe immunosuppression contrary to what was reported in South Africa where the authors proposed not to use the test (100). In another study in Tanzania, against culture-confirmed pulmonary TB the sensitivity was 50% among HIV-infected children with a high specificity of 97.1%(179). The urine LAM test has a fair sensitivity but with poor specificity. Although the manufacturer instructions indicate that the test is specific for MTB, our findings indicate that contamination from the skin during collection can hamper the test sensitivity. This is shown by the fact that most of the false reactions occurred in samples with culture growth while cleaning with antibiotic solution improved the specificity. Our study was one of the very few studies evaluating the urine LAM test not only in HIV infected children. However, the sensitivity was not better among HIV infected children and close to what was reported among -HIV infected adults but with a lower specificity.

Reasons for poor specificity are diverse. One study in neighboring Tanzania reported environmental factors including dust, soil, and fecal matter, as potential contaminants likely to

cause poor specificity for urine LAM although, blood, bacterial or fungal organisms, had no effect on the results(179). Others have attributed contamination in adult tests to oral flora including candida species and non-tuberculosis mycobacteria that may find their way into the sample at collection(180–182). Urine sample collection for urine LAM testing should therefore be strict in order to avoid contamination and allow better utility. However, this approach alone might not be sufficient since in our study most children had urine collected using catheter.

Despite this challenge of contamination, we note that the lack of proper reference standard could have also limited the potential value of this test and underestimated the specificity. Indeed the paucibacillary nature of respiratory samples and the expectation that the urine LAM is mostly useful to diagnose the disseminated TB cases who are more likely to have few bacilli in their respiratory specimen, the microbiological reference standard based on respiratory samples might be even less adequate for evaluating new test for diagnosis of disseminated TB. This could also potentially explain why false positive seems to be more common among low grade LAM positive. On the other hand, the false positive results do not seem to be due to low reading reliability since the inter-reader reliability was very high with kappa above 0.8

The major study limitation was that the study population had fewer confirmed cases than expected and this could affect any accuracy test. Another limitation was the poor yield of culture, despite the use of two different culture methods. MGIT, which is the most sensitive TB test method missed to pick some Xpert-positive results despite children being naïve for anti-tuberculosis drugs. If the reference standard is weak, this could contribute to the low specificity of the urine LAM. At the same time, this low yield may mislead by over estimating the sensitivity of Xpert. On the other hand the reference standard was strengthened by the combination of Xpert, culture from respiratory samples and other samples except stool.

Finally, a high proportion of children had been on antibiotics for treating cough. We do not think this had a significant impact on the TB culture outcomes as children most likely were not exposed to quinolones.

Urine LAM has a strong advantage as it uses a sample that is easy to collect, less contaminated than sputum and offers rapid results especially for patients with high risk of mortality that require immediate initiation of TB treatment. Therefore, more optimization needs to be done to improve the test. Indeed, there is prospect of a newer and improved LAM point of care test, known as Fujifilm SILVAMP TB LAM (FujiLAM). Already emerging data from frozen samples in HIV infected adults indicates sensitivity of 70.4% (53- 83.1) and specificity of 90.8% (86-94·4). This sensitivity is 28% higher for the FujiLAM than the AlereLAM but with a 4% lower sensitivity(183). More field evaluations are needed to determine the accuracy and feasibility of this new version that could revitalize childhood TB diagnosis. Hence the search continues to determine more sensitive point of care tests that will help in diagnosing TB particularly in critically ill children.

3.3.6 Involvement in this work

In this study my involvement was in the design of the protocol, mainly the laboratory component. And as a full time TB laboratory responsible, I was overseeing and coordinating the inter lab and intra laboratory activities of the study. In addition, I coordinated development of tools such as analytical plans, procedures and other document that were used to capture data from the laboratory. Besides that, I supervised the laboratory activities to ensure that they were performed according to the good clinical and laboratory practice as well as laboratory quality management system. I was also involved in data interpretation and manuscript writing.

Accuracy of two non-sputum based tuberculosis diagnostic tests (Xpert MTB/RIF in stool and urine LAM) in children at increased risk of severe or disseminated tuberculosis

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Background and objective

- In 2017, approximately 1 million children below 15 years developed TB but only 45% were notified (WHO 2018)
  - 15% of total deaths
  - 10% of total deaths in HIV positive
- Diagnosis remains a major challenge due to difficulty to collect respiratory specimen in children and due to the paucibacillary form of TB in children
- Majority of children are started on empirical TB treatment based on presumptive TB.
- Young children or children with comorbidity like HIV and malnutrition are more likely to present severe form and disseminated form of TB with poor prognosis and are even more difficult to diagnose.
- The urine lipoarabinomanan (LAM), is an easy to use point of care test recommended in advanced HIV patients with CD4 <100.
- Few studies have evaluated the performance in children.
- We assessed the diagnostic accuracy of two non-sputum based tests, Xpert MTB/RIF in stool and urine LAM, in children at increased risk of severe or disseminated TB in Uganda

Methods

Study site: Mbarara, South West Uganda

Study population

- Clinical suspicion of TB and either age< 2 years, HIV infected, severely malnourished or severe pneumonia
- Informed consent was administered to participants

Laboratory procedures

- Xpert MTB/RIF and TB culture were performed on the following:
  - one gastric aspirate
  - one nasopharyngeal aspirate (or sputum)
  - necropsy samples when available,
  - two stool samples
  - Extra-pulmonary samples; based on clinical presentation
- Blood culture samples on children with fever (38°c)
- Urine LAM Point of care test was done on two urine samples
- TB culture was performed using Nalc-NaOH at a final concentration of 1.5% for stool and necropsies and 1.25% for the others
- Samples were neutralised with phosphate buffer at PH of 6.8
- After centrifugation, sediments were mixed with 2ml of buffer and inoculated on two Lowenstein Jensen media and one MGIT per sample.

TB reference standard for the accuracy of Xpert on stool and LAM was:

- **Positive**: MTB culture or Xpert result positive from any sample collected except stool.
- **Negative**: one negative culture or Xpert result from at least two different samples (2 respiratory or 1 respiratory and another sample) without any positive result

Results

- 234 children enrolled;
  - 161 (68.8%) were < 2 years,
  - 73 (31.2%) HIV infected,
  - 15 (6.4%) had TB contact history,
  - 115 (49.1%) severe malnutrition
  - 66 (28.2%) severe pneumonia
- TB confirmed in 12 cases (5.1%): 10 from respiratory specimens and 2 from necropsy samples
- TB detected by Xpert in stool in 9/233 (3.8%) cases
- Urine LAM was positive with grade two or higher in 17/231 patients (7.4%)

Accuracy results

	SE	SP	PPV	NPV
Overall				
2 LAM (1+ cutoff)	6/12 (50.0%)	155/210 (73.8%)	6/61 (9.8%)	155/161 (96.3%)
2 LAM (2+ cutoff)	5/12 (41.7%)	199/210 (94.8%)	5/16 (31.3%)	199/206 (96.6%)
2 stool	6/12 (50.0%)	211/213 (99.1%)	6/8 (75.0%)	211/217 (97.2%)
HIV+				
2 LAM (1+ cutoff)	2/4 (50.0%)	49/63 (77.8%)	2/16 (12.5%)	49/51 (96.1%)
2 LAM (2+ cutoff)	1/4 (25.0%)	60/63 (95.2%)	1/4 (25.0%)	60/63 (95.2%)
2 stool	1/4 (25.0%)	65/65 (100.0%)	1/1 (100.0%)	65/68 (95.6%)
Severe acute malnutrition				
2 LAM (1+ cutoff)	4/5 (80.0%)	105/144 (72.9%)	4/43 (9.3%)	105/106 (99.1%)
2 LAM (2+ cutoff)	4/5 (80.0%)	135/144 (93.8%)	4/13 (30.8%)	135/136 (99.3j%)
2 stool	3/5 (60.0%)	145/147 (98.6%)	3/5 (60.0%)	145/147 (98.6%)

Investigations of false positive LAM results as compared to true positive LAM results

	False Positive LAM	True Positive LAM
% grade 1	44/55 (80.0%)	1/6 (16.7%)
Bacterial pathogen in urine	16/50 (32.0%)	1/6 (16.7%)
Only 1 of 2 LAM+	27/55 (49.1%)	1/6 (16.7%)

Discussion and Conclusion

- Proportion of confirmed TB was very low among these sick children.
- Xpert in stool identified half of the cases and could be a good option in these children.
- The low specificity of the urine LAM requires preventing its recommendation for TB diagnosis in children
  - Trend of more bacterial pathogens in urine of patients with FP LAM result
  - Higher proportion of low grade results in FP LAM results
    - Potential reading errors between negative and 1+ to be investigated
    - Use of higher grade positive cut-off?

Chapter 3.4

Evaluation of Omnigene®-Sputum and ethanol for preservation of sputum prior to Xpert and culture

3.4.1 Justification and objectives

As described in previous chapters, some of the best diagnostic tests, in most low resource settings are found at intermediate or national reference laboratories. Because of that, samples that require to be tested for drug resistance or further advanced testing have to be transported to these facilities. Most of these low resource settings have challenges of infrastructure, power and often experience breakdown in the cold chain system during transportation. This often creates high temperature conditions on transit that may result in poor diagnostic yield from samples as a result of high contamination, poor growth due to loss of viability and sometimes false negative outcomes.

In order to overcome these challenges, a simple but robust system that allows samples to be transported at room temperature conditions longer than the recommended time prior to testing with the most reliable methods like Xpert and culture would be a very useful solution. Indeed, according to manufacturer's instruction, specimens to be tested on Xpert should be held at 2-8 °C for 10 days maximum or be stored at a maximum of 35°C for up to 4 days before processing(128). TB culture equally requires a fresh sample or refrigeration at 2-8 °C not beyond 4 days (184).

In the absence of a cold chain, sputum preservation for culture currently relies on cetylpyridinium chloride, a validated and affordable solution, but only favors laboratories that use solid media. Omnigene (OM-S; DNA Genotek Inc, Ottawa, Canada) reagent is believed to preserve sputum before testing with both Xpert and MGIT culture. Its advantage lies in ability to stabilize samples and DNA for a maximum of 30 days at room temperature before Xpert and 8 days before culture. It also reduces the processing steps, especially for culture and because of reduced steps avoids possibility of errors and infection control challenges. Further evaluation with Xpert and MGIT culture was required due to inconsistent results from previous studies(136,142–145,185,186).

Similarly, ethanol is a low-cost and effective method of DNA preservation before PCR testing.

Objectives

- To determine the effect of OM-S and ethanol preservation on sputum samples tested with Xpert after 15 days
- To assess OM-S on samples tested with MGIT culture after 8 days

- To investigate the effect of delayed Xpert and MGIT culture testing beyond recommended times for untreated sputum samples.

3.4.2 Methods, results and conclusion

Methods

The study enrolled newly diagnosed adult patients with known sputum smear-positive results from the MRRH laboratory.

The study was done in two independent phases. In phase one, three sputum samples from consenting patients were collected at 1 hour interval by a nurse in the outpatient department. Quality of samples was checked and samples were pooled, split into five aliquots for different evaluations. At each stage, smear microscopy was performed to ensure homogeneity of the samples. Three of the split samples were tested using Xpert, without any preservative, at day 0, 7 and 15. The other two samples were added with OM-S or ethanol and left to stand at room temperature until testing at day 15. All samples were kept at room temperature on the laboratory bench.

In phase two, we collected at most 3 samples from each patient in order to achieve the required volume for the aliquots to test for culture. Microscopy evaluation was done for homogeneity and then samples were split into four aliquots. Two of the aliquots were kept at room temperature without any preservative and tested on day 0 and 8. The other two were preserved with OM-S, kept, decontaminated and cultured on day o and 8 as the first ones.

At analysis, the ability of the different reagents/preservatives to preserve and decontaminate samples prior to testing with Xpert and culture was assessed by calculating positivity rate for the two methods.

The study documented that by the 15th day, there was no major difference in Xpert detection between samples treated with OM-S, ethanol and samples tested at day 0 without any preservative. However, in the study conditions, there was also no difference in Xpert detection on untreated samples tested at day 0 and day 15. The benefit of preserving samples with either OM-S or ethanol

was overshadowed by the recovery of TB in non-preserved samples for 15 days. The study recommended further evaluation under routine field laboratory conditions to assess the reproducibility of the study results. In the second phase, we observed a substantial loss of viability of MTB on samples treated with OM-S and tested by MGIT culture at both day 0 and day 8 of the evaluation, and this does not support the use of OM-S for sample transportation before MGIT testing. We recommend further optimization of the OM-S reagent for future transportation and preservation of sputum samples for MGIT culture. **(Further details of this work as per the attached manuscript accepted for publication by Journal of Clinical Microbiology).**

3.4.3 Involvement in this work

In this study my involvement was in the entire process of protocol development and submission for institutional and national ethical approvals. In addition, I coordinated development of analytical plans, procedures and other document that were used to capture data from the laboratory. Besides that, I supervised the laboratory activities to ensure that they were performed according to the good clinical and laboratory practice as well as laboratory quality management system. I took lead in data analysis and manuscript writing and submission to the appropriate JCM. Finally, the manuscript was accepted for publication as shown in full publication in next page.

Evaluation of OMNIgene® SPUTUM and ethanol reagent for preservation of sputum prior to Xpert and culture testing in Uganda

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Abstract

Background: Xpert MTB/RIF (Xpert) and culture are the most reliable methods for tuberculosis diagnosis but are still poorly accessible in many low resource countries. We aimed to assess the effect of OMNIgene® SPUTUM (OM-S) and ethanol in preserving sputum for Xpert and OM-S for mycobacteria growth indicator tube (MGIT) testing over a period of 15 and 8 days respectively.

Methods: Sputum were collected from newly diagnosed smear-positive patients. For Xpert, pooled samples were split into 5 aliquots: 3 for Xpert on day 0, 7 and 15 days without additive and 2 with either OM-S or ethanol at day 15. For MGIT, 2 aliquots were tested without preservative and 2 with OM-S at 0 and 8 days.

Results: A total of 48 and 47 samples were included in the analysis for Xpert and culture. With Xpert, using Day 0 as reference, untreated samples stored for 7 and 15 days showed concordance of 45/46 (97.8%) and 46/48 (95.8%). For samples preserved with OM-S or ethanol for 15 days compared with untreated samples processed at day 0 or after 15 days, OM-S concordance was 46/48(95.8%) and 47/48(97.9%), while ethanol was 44/48 (91.7%) and 45/48 (93.8%). With MGIT, concordance between untreated and OM-S treated samples was 21/41(51.2%) at Day 0 and 21/44(47.7%) at day8.

Conclusions: Xpert equally detected TB in OM-S treated and untreated samples up to 15 days but showed slightly lower detection in ethanol treated samples. Among OM-S treated samples, MGIT positivity was significantly lower compared to untreated samples at both time-points.

Key words: OMNIgene®, Tuberculosis, Xpert, Culture

Introduction

Tuberculosis (TB) represents one of the most prevalent infectious diseases in the world, with an estimate of 10 million incidence cases in 2017, majority from low or middle income countries (1). In 2010, World Health Organization (WHO) endorsed Xpert MTB/RIF (Xpert) (Cepheid, Sunnyvale, CA), to simultaneously detect TB and resistance to rifampicin (2) and the test has been widely adopted for TB diagnosis (1). Nevertheless Xpert remains unavailable in most primary health care centres where majority of patients with presumptive TB seek care (3). Culture is the gold standard test to confirm TB, but is slow, laborious, and due to requirement for biohazardous containment, is available mainly in high level laboratories. With Xpert placed at district hospital and culture at regional hospital and national reference laboratory in many low resource countries, sputum samples must be transported from peripheral locations for testing. In some remote setting, high temperatures and long transport make proper samples storage very challenging.

According to manufacturer's instruction, specimens to be tested on Xpert should be held at 2-8 °C for 10 days maximum or be stored at a maximum of 35°C for up to 3 days before processing (4). Even if these limitations hinder access to Xpert, studies on stability of samples prior to Xpert testing are limited. Fixation of samples with ethanol is a low-cost and effective method of DNA preservation before PCR testing, (6) however data on its application on samples before Xpert testing are not available. Samples for culture should be processed immediately or kept at 2-8 °C not beyond 3 days.

Long sample storage before culture inoculation is known to increase contamination rate and affect mycobacterial recovery (7). Cetylpyridinium chlorite is a sample preservative widely used for sample transportation, but this reagent is not compatible with the mycobacteria growth indicator tube (MGIT) technique, commonly used for TB culture (8).

OMNIgene® SPUTUM (OM-S; DNA Genotek Inc, Ottawa, Canada) is another reagent that can be applied to samples prior to testing with both Xpert and MGIT cultures. The reagent stabilizes DNA prior to PCR testing, so that samples treated with OM-S may be stored for a maximum of 30 days at a temperature between 4 and 40 °C before Xpert testing (DNA Genotek procedures). One

study reported good compatibility of OM-S with Xpert in samples transported at room temperature (RT) compared to standard procedures including cold storage (5). However, this study did not systematically compare Xpert performance on OM-S with standard method for the same duration of storage.

At the same time OM-S has the ability to liquefy and decontaminate samples offering the possibility to extend their storage until 8 days at temperatures up to 40°C prior to culture inoculation (9). However, studies investigating the effect of OM-S have shown good accuracy but mainly with Löwenstein-Jensen culture (10,11) while those using MGIT have reported contrasting results (12–15).

The objectives of this proof of concept study were: to determine the effect of OM-S and ethanol when added to samples tested with Xpert after 15 days; to assess OM-S on samples tested with MGIT culture after 8 days; to investigate the effect of delayed Xpert and MGIT culture testing beyond recommended times for untreated sputum samples.

Materials and Methods

Setting

The study was conducted at Epicentre Mbarara Research Centre, within a Regional Referral Hospital in south western Uganda. The biosafety level 3 Epicentre laboratory is quality controlled by the Supranational TB Reference Laboratory of the Tropical Medical Institute of Antwerp (Belgium).

Sample collection

Xpert and MGIT performance were investigated in Phase 1 and Phase 2 of the study among newly diagnosed smear positive (Sm+) adults.

Sm+ patients identified under routine of care were referred for informed consent and enrolment at the Epicentre Clinic, where 1 to 3 samples (A,B,C) were collected within 1-hour interval, to reach at least 6 ml total volume for the first phase and 10 ml for the second phase. Samples were pooled

to obtain a homogenous bacterial load before splitting in aliquots for the different testing strategies. To verify homogeneity, smear microscopy using auramine staining according to WHO/IUATLD AFB microscopy grading (16) was performed on direct, pooled sample and on all the aliquots. Smear-negative (Sm-) pooled samples and insufficient volume samples were excluded from further evaluation. All aliquots were stored at RT between 22-26oC in a temperature-controlled laboratory throughout the study investigation period.

Sample processing and testing

Phase 1: assessment of the effect of OM-S and ethanol on the Xpert test

Pooled samples were split into five equal aliquots: a) three additive-free, one tested on the collection day, one after 7 and one after 15 days respectively; b) two treated with either OM-S or ethanol and tested after 15 days (Fig 1A).

Aliquots treated with either OM-S or ethanol were added to the preservative in equal volume. Then, 1 ml of the mixture was combined with 2 ml of sample reagent, mixed and allowed to settle for 15 minutes at RT before transferring 2 ml into the Xpert cartridge for testing according to the manufacturers' protocol (4).

Phase 2: assessment of the effect of OM-S on MGIT culture

Pooled samples were split into four equal aliquots: a) two untreated: one tested on the collection day and another after 8 days, b) two aliquots added with OM-S and processed on collection day and after 8 days (Fig 1B).

Aliquots treated with OM-S were added with the reagent in 1:1 proportion following manufacturer instructions (4), inverted vigorously and left at RT. On the scheduled day for culture inoculation, the mixture was centrifuged at 3,000xg for 20 minutes, the supernatant was discarded, and the sediment suspended into 1 ml of phosphate buffer before inoculation into an MGIT tube. Untreated aliquots were decontaminated with 1.25% N-acetyl L-Cysteine-Sodium hydroxide final concentration, then centrifuged at 3,000xg for 20 minutes. The pellet was re-suspended with 2 ml of phosphate buffer and inoculated into MGIT. PANTA (Polymyxin B, Amphotericin B, Nalidixic

acid, Trimethoprim, Azlocillin) was used at double concentration according to a modified step of the BD MGITTM product insert (17).

Positive cultures were checked for AFB presence using Ziehl-Neelsen microscopy and tested on blood agar culture to exclude contamination (17). Final identification of Mycobacterium tuberculosis complex (MTB) was performed using MPT64 (SD Bioline)-Rapid Diagnostic Test. Cultures were classified as negative after 8 weeks of incubation.

Statistical analysis

A convenient sample size of 50 Sm+ TB patients was proposed for each phase of the study. Laboratory records were double entered into voozanoo database and analysed using STATA 12 (Texas, USA), software.

Xpert results were categorized as very low/low; medium/high, negative/not applicable (inconclusive results; either error, invalid, no result). Results were presented per stratified aliquot smear results grouped as: low ($\leq1+$) and high bacillary load ($>1+$).

To assess the effect of time alone (without preservative) on test performance, MTB detection on Xpert was compared between day 0, day 7 and day 15. To assess the effect of both preservatives, MTB detection on Xpert was compared between aliquots treated with OM-S and ethanol at day 15, and each method versus untreated aliquots at day 0 and day 15. Xpert results were considered discordant between aliquots if the difference was exceeding one grade of positivity.

MGIT positivity rate was stratified by smear categories: negative, low ($\leq1+$) and high bacillary load ($>1+$). To assess the effect of time alone, untreated samples were compared at day 0 and day 8. To assess the effect of OM-S on MGIT, OM-S treated aliquots at day 8 were compared to untreated aliquots at day 0 and day 8 along with OM-S at day 0. To investigate the effect of OM-S on mycobacterial viability, treated and untreated aliquots were compared at day 0.

Finally, median time to culture positivity and its interquartile range (IQR) was calculated in days among untreated and OM-S treated aliquots at different time points.

Ethical approval: Approvals were received from Mbarara University Research Ethics Committee, the Uganda National Council for Science and Technology and ITM Ethical Review Board.

Results

Phase 1: assessment of the effect of OM-S and ethanol on the Xpert test

Between May 2016 and October 2017, the study enrolled 52 patients in phase 1. Of these, 2 submitted insufficient sample volume and were excluded. Fifteen patients (30%) provided 6ml sample, which did not require additional sample collection, 32 (64%) 2 samples and 3 (6.2%) 3 samples, for total of 88 samples. After pooling samples, 2/50 (4%) aliquots were Sm- and excluded from further analysis. Of 48 remaining samples, 10 (20.8%) were scanty positive, 14 (29.2%) 1+, 10 (20.8%) 2+ and 14 (29.2%) 3+. All aliquots obtained from the same sample showed either the same grade of positivity or 1 grade level of difference except for 5 samples (ID107, 115, 140, 144, 145), (Table 1).

MTB was detected by Xpert in all aliquots except for 2 invalid results for 1+ untreated aliquots tested at day 7 (ID 109 and 144), and 2 negative results for aliquots treated with ethanol; one Sm- and one scanty positive (ID 115 and 120) (Table 2).

Xpert performance for untreated specimens

When we compared untreated aliquots obtained from the same sample and tested at day 0, 7 and 15, except for two samples (ID 120,153) aliquots varied within one degree of positivity (Table 3). Aliquot 120 was "high" at day 0 but "low" at all other time points. On the contrary, aliquot 153 was "very low" at day 0 and "medium" at day 15.

Using day 0 as reference and excluding invalid results, 45/46 (97.8%) aliquots had concordant results with those of day 7, while 46/48 (95.8%) with those of day 15.

Effect of OM-S and ethanol specimen treatment on Xpert performance

The results from the comparison between aliquots tested with OM-S or ethanol and versus untreated aliquots at day 0 and day 15 is shown in Table 4.

Three aliquots (ID 120,152,153), showed discordant Xpert results in the OM-S group. Aliquot 120 showed a lower grade of positivity with OM-S compared to day 0 without treatment, while aliquot 152 and 153 had higher grade with OM-S compared to untreated at day 0 and day 15.

OM-S aliquots had Xpert concordant results with untreated aliquots in 46/48 (95.8%) and 47/48 (97.9%) at day 0 and day 15, respectively.

Five aliquots (ID 152, 153,120,142,144) showed discordances in the ethanol group. The aliquots 120, 142,144 added with ethanol gave lower results compared to untreated aliquots at both time points, while aliquots 152 and 153 reported "high" Xpert results with ethanol but "low" or "very low" when untreated. Of 48 aliquots containing ethanol, 44 (91.7%) and 45 (93.8%) had concordant results with untreated aliquots tested at day 0 and day 15, respectively (Table 4).

Comparison of aliquots treated with OM-S and ethanol showed a concordance of 44/48 (91.7%) (Table 5). Two aliquots were positive for OM-S and negative with ethanol (ID 120,115), and two (ID 142,144), were "high" positive with OM-S and "low" or "very low" with ethanol.

All aliquots gave rifampicin susceptible results except for ID 120 that was rifampicin resistant for untreated aliquots at day 7, 15 and with OM-S, but rifampicin susceptible at day 0 and negative for the aliquot treated with ethanol.

Phase 2: assessment of the effect of OM-S on MGIT cultures

Of 57 patients enrolled in phase 2 between October 2016 and August 2017, 1 patient was excluded because of insufficient sample volume. Of 56 patients finally included, 33 (62%) provided one 10 ml sample and 23 (38%) collected 2 sputum samples, and none required a third sample. Of the 56 pooled samples, 8 were excluded because Sm-, the remaining 48, had 5 (10.4%) scanty, 18 (37.5%) 1+, 10 (20.8%) 2+, 15 (31.2%) 3+. All aliquots prepared from the same pooled sample showed either the same level of smear positivity or 1 grade level of difference, except for 4 cases (ID 206, 208, 230, and 236) (Table 6).

For sample ID242 the untreated aliquot at day 0 was contaminated, the untreated aliquot at day 8 was not tested, while the other aliquots were smear and culture negative. One aliquot (ID 240 untreated day 8) was positive for non-tuberculous mycobacteria (NTM).

As shown in Fig 2, the culture positivity across smear categories at different time points was uniformly distributed.

MGIT performance for untreated specimens

At day 0, 41/47 (87.2%) untreated aliquots had MTB culture positive results compared to 44/46 (95.7%) at day 8.

Effect of OM-S specimen treatment on MGIT performance

Untreated and OM-S treated aliquots were compared at day 0 and day 8. Of the 41 untreated aliquots with MTB at day 0, only 18 (43.9%) treated with OM-S had MTB at day 8 (Table 7). Similarly among 44 MTB+ untreated cultures at day 8, merely 20 (45.5%) were positive among OM-S treated aliquots the same date (Table 7). In addition, among 21 MTB+ OM-S treated aliquots at day 0, only 11 (52.4%) were positive among OM-S treated at day 8 (Table 7).

By comparing OM-S treated aliquots at day 0 and day 8, there were 11 MTB+ cultures at both time points, 10 at day 0 and 9 at day 8 alone (Table 7).

Finally, among 41 MTB+ untreated aliquots at day 0, only 21 (51.2%) were positive among OM-S aliquots the same date (Table 8).

Time to culture positivity for OM-S and untreated samples at different time points

At day 0, median time to detection was 8 days (IQR, 5- 15) among untreated aliquots compared to 13 days (IQR, 11-35) for OM-S. Correspondingly, it was 11 days (IQR: 6-35) at day 8 among untreated aliquots compared to 20 days (IQR: 15-42) for OM-S.

DISCUSSION

OM-S has been proposed as sample preservative prior to testing with Xpert and culture, but so far has not been endorsed by WHO (18). This study adds more evidence of accuracy on the use of this reagent to preserve samples for delayed testing. The study also provides data on Xpert and MGIT performance on samples kept beyond the recommended 3 days at RT without preservative. Samples treated with OM-S can be stored up to 30 days at RT prior Xpert testing. Our choice to limit the delay for a maximum of 15 days was based on assumption that the benefit of this test is to provide early diagnosis, and would be compromised if results are available beyond this time frame.

Overall, all aliquots gave Xpert positive results with the exception of 4 aliquots: 2 treated with ethanol and 2 untreated at day 7 with instrument errors; code 2008 and 5007 linked with smear grade 1+. These errors are reported by the Cepheid as mainly related to high pressure and probe check control failure so they are mainly due to specimen handling rather than RT preservation (21). Surprisingly, the effect of long sample storage at RT without a preservative did not alter the Xpert performance over 15 days. Only 2 aliquots showed Xpert quantitative result discordant for more than one grade. These results suggest that mycobacterial organisms in Sm+ samples may not significantly degrade by storage beyond the 3 recommended days.

There was a good concordance between aliquots added with OM-S and untreated tested at day 0 and 15. This shows that OM-S does not alter the Xpert performance on specimen stored up to 15 days at RT, compared to testing at day 0 that is considered as the best practice. It also demonstrates that the reagent did not improve MTBc detection after long storage compared to untreated samples. At the same time points, ethanol performance was lower, with 5 discordant results. However, with exception of two aliquots that were either Sm- or scanty, and Xpert negative, all aliquots treated with ethanol gave positive results.

In both OM-S and ethanol comparisons, all discordances (results above one grade difference) occurred in 5 samples (ID 120,142,144,152,153) for which however smear microscopy grading did not show difference between aliquots. For ID 120 the same aliquot showed discordance with

rifampicin result: Xpert positive and rifampicin susceptible at day 0 untreated but resistant for extended untreated aliquots at day 7 and 15, ethanol and OM-S both at day 15. This could be due to a clerical error from the laboratory, but other explanations cannot be excluded, such as heteroresistance or false susceptible result due to low mycobacterial load, as reported by other studies (19, 20). However, this discrepancy was not further investigated.

Other studies have reported already good performance of Xpert from OM-S treated compared to untreated samples but always processed on the same day of collection (5,12). In addition, our study showed that similar performance can be obtained beyond the recommended time with OM-S treated and untreated samples until 15 days. Although Xpert testing should be performed as soon as the sample is collected to allow rapid treatment initiation, these results are very important for remote settings where Xpert can only be tested after prolonged transport collection.

MGIT performance was much lower for samples treated with OM-S compared to untreated samples (50%). The poor concordance at day 0 indicates a negative effect on bacterial growth of MTB by the OM-S treatment regardless of time of exposure. Genotek has recently released a revised protocol that includes OM-S neutralization with buffer before inoculation. This procedure should be further investigated.

The negative effect of OM-S on mycobacterial recovery on MGIT has been reported in other studies (13,14). The incompatibility between the reagent and culture however has been mainly reported for MGIT system (16,13,17). One study reported poor recovery of MTB across both MGIT and LJ media (13). One study reported improved results in MGIT cultures using samples treated with OM-S for up to three weeks, with only concerns about delay in MGIT results (15).

Other studies have reported no significant difference between untreated and OM-S treated smear positive remnant samples, with MGIT at day 8(18). Although there was a difference in study design, our study used fresh samples, while FIND evaluation used sediments, this is unlikely to have caused such a difference in the results.

There was only one contaminant on untreated sample at day 0. Previous studies have shown that OM-S treated samples have lower contamination rate than untreated counterparts (10,12–15). In

our study, only one contaminant in the untreated group may not explain much about the contribution of OM-S in reducing contamination compared to standard decontamination.

Finally, we observed a substantial delay in days to positivity between untreated and OM-S treated samples at both time points. Previous studies have also noted delayed culture growth in samples treated with OM-S (13,15,23,24). This further raises concerns about the utility of OM-S in its current procedure and the compatibility with MGIT cultures.

Limitations

This study had few limitations. This was a proof of concept study and aliquots were stored in a controlled research laboratory and not in the type of setting in which the protocols would actually be applied.

We used only known Sm+ samples and therefore we could not demonstrate the effect of the reagent in Sm- samples tested on Xpert and in MGIT liquid medium. More evaluation is needed especially among smear negative, Xpert positive samples in high TB-HIV context.

Conclusion

In this proof of concept study, we have shown that there is no advantage in using OM-S reagent, or ethanol, for smear positive sputum stored at RT up to 15 days as Xpert performance remains high even after such delays. This study brings reassuring data regarding the possibility of using Xpert on transported sputum samples without cold chain, which is common practice in high burden and limited resource countries. On the other hand, this study confirms the need to perform MGIT culture on fresh sputum samples and confirm WHO recent position to not support the use of OM-S for delayed culture processing, unless additional evaluation on the revised protocol give more promising results.

Acknowledgment

References

1.   WHO. 2017. Global Tuberculosis Report 2018. Vol. 69, Pharmacological Reports. 683-690 p.

2.   WHO. 2012. Xpert MTB / RIF Test: WHO endorsement and recommendations.

3.   WHO. 2011. Automated real-time nucleic acid amplification technology for rapid and simultaneous detection of tuberculosis and rifampicin resistance: Xpert MTB/RIF. Policy statement.

4.   Cepheid. 2009. Operator Manual test Xpert MTB / RIF.

5.   Maharjan B, Weirich A, Curry PS, Hoffman H, Avsar K, Shrestha B. 2016. Evaluation of OMNIgene W SPUTUM-stabilised sputum for long- term transport and Xpert W MTB / RIF testing in Nepal. Int J Tuberc Lung Dis. 1;20(12):1661-1667.

doi: 10.5588/ijtld.16.0421.

6.   Williams DL, Gillis TP, Dupree WG. 1995. Ethanol fixation of sputum sediments for DNA-based detection of Mycobacterium tuberculosis. 33(6):1558-61.

7.   Paramasivan CN, Narayana ASL, Prabhakar R, Rajagopal MS, Somasundaram PR, Tripathy SP. 1983. Effect of storage of sputum specimens at room temperature on smear and culture results. Tubercle. 64(2):119-24. http://dx.doi.org/10.1016/0041-3879(83)90036-3

8.   Siddiqi SH. 2006. MGIT TM Procedure Manual for BACTEC TM MGIT 960 TM TB System. System.

9.   DNAgenotek. 2018. Omigene Sputum Flyer. 1–2.

10.   Affolabi D, Sanoussi N, Sossou A, Nys T, Bodi O, Esse M, Houeto S, Massou F, de Jong BC, Rigouts L. 2018. Performance of OMNIgene•SPUTUM (DNA Genotek) and cetylpyridinium chloride for sputum storage prior to mycobacterial culture. J Med Microbiol. 67(6):798–805. doi: 10.1099/jmm.0.000745.

11.   Maharjan B, Shrestha B, Weirich A, Stewart A, Kelly-Cirino CD. A novel sputum transport solution eliminates cold chain and supports routine tuberculosis testing in Nepal. J Epidemiol Glob Health. 2016;6(4):257–65. http://dx.doi.org/10.1016/j.jegh.2016.04.002

12.   Kelly-Cirino CD, Musisi E, Byanyima P, Kaswabuli S, Andama A, Sessolo A, Sanyu I, Zawedde J, Curry PS, Huang L. 2017. Investigation of OMNIgene·SPUTUM performance in delayed tuberculosis testing by smear, culture, and Xpert MTB/RIF assays in Uganda. J Epidemiol Glob Health. 7(2):103–9. http://dx.doi.org/10.1016/j.jegh.2017.04.001

13.   Zallet J, Olaru ID, Witt A, Vock P, Kalsdorf B, Andres S, Hillemann D, Kranzer K. 2018. Evaluation of OMNIgene SPUTUM reagent for mycobacterial culture. Int J Tuberc Lung Dis. 1;22(8):945-949. doi: 10.5588/ijtld.17.0020.

14.   Azam K, Cadir N, Madeira C, Gillespie SH, Sabiiti W. 2018. OMNIgene SPUTUM suppresses contaminants while maintaining Mycobacterium tuberculosis viability and obviates cold-chain transport. ERJ Open Res. 16; 4(1):00074-2017.

doi: 10.1183/23120541.00074-2017.

15.   Tagliani E, Alagna R, Tafaj S, Hafizi H, Cirillo DM. 2017. Evaluation of Mycobacterium tuberculosis viability in OMNIgene-SPUTUM reagent upon multi-day transport at ambient temperature. BMC Infect Dis. 17(1):663. doi: 10.1186/s12879-017-2756-3.

16.   Rider HL, Van Deun A, Kam KM, Kim SJ, Chonde TM, Trébucq A, Urbanczik R. 2007. Priorities for tuberculosis bacteriology services in low-income countries. 2nd edition. International Union Against Tuberculosis and Lung Disease, 68 boulevard Saint Michel, 75006 Paris, France

17.   Siddiqi SH. 2006. MGIT For BACTEC TM MGIT 960 TM TB System. FIND MGIT Manual.

18.   World Health Organization (WHO). 2017. Commercial products for preserving clinical specimens for the diagnosis of tuberculosis. Bulletin.

19.   Van Rie A, Mellet K, John MA, Scott L, Page-Shipp L, Dansey H, Victor T, Warren R. 2012. False-positive rifampicin resistance on Xpert® MTB/RIF: Case report and clinical implications. Int J Tuberc Lung Dis. 16(2):206-8. doi: 10.5588/ijtld.11.0395.

20.   Kelly JD, Lin SYG, Barry PM, Keh C, Higashi J, Metcalfe JZ. 2014. Xpert MTB/RIF false detection of rifampin-resistant tuberculosis from prior infection. Am J Respir Crit Care Med. 1;190(11):1316-8. doi: 10.1164/rccm.201408-1500LE

21.   Cepheid. 2012. GeneXpert System; Operational Manual. Cepheid. (301–0045–Rev C version4).

22.   Asandem DA, Asante-Poku A, Asare P, Aboagye SY, Stephen OW, Danso E, Klevor PM, Hayibor KM, Yeboah-Manu D. 2018. OMNIgene SPUTUM: A good transport and decontaminating reagent for tuberculosis testing. Int J Mycobacteriol. 7(3):222-227.   doi: 10.4103/ijmy.ijmy_102_18.

23.   Kelly-Cirino CD, Curry PS, Marola JL, Helstrom NK, Salfinger M. 2016. Novel multi-day sputum transport reagent works with routine tuberculosis tests and eliminates need for cold chain: Preliminary study of compatibility with the Xpert® MTB/RIF assay.

Diagn Microbiol Infect Dis. 86(3):273–6.

24.   WHO. 2011. Commercial serodiagnostic tests for diagnosis of tuberculosis: policy statement. Geneva WHO.

Figure 1 A. Phase 1: *assessment of the effect of OM-S and ethanol on the Xpert test*

Sputum one: (A) — Sputum two: (B) — Sputum three: (C)

Perform smear microscopy on each Sample

Pool samples in 50ml tube and perform microscopy on smear

- Aliquot A; Untreated → Keep at RT → Add 1ml sample to 2ml reagent → Perform Xpert MTB/ Rif assay same day
- Aliquot B; Untreated → Leave at RT for 7 days → Add 1ml sample to 2ml reagent → Perform Xpert MTB/ Rif assay
- Aliquot C; Untreated → Keep at RT
- Aliquot D; OMNIgene treated → Add equal volume of Omnigene to sample
- Aliquot E; Ethanol treated → Add double volume of ethanol to sample

Leave for 15 days → Add 1ml sample to 2ml reagent → Perform Xpert MTB/ Rif assay

Figure 1B. Phase 2: *assessment of the effect of OM-S on MGIT culture*

Sputum one: (A) — Sputum two: (B) — Sputum three: (C)

Perform smear microscopy on each Sample

Pool samples in 50ml tube and perform microscopy on smear

- Aliquot A: Untreated → Keep at RT → Add 1%NAOH to equal vol. sample → Use sediment to inoculate MGIT same day
- Aliquot B: Omnigene treated → Add Omnigene and Keep at RT → Centrifuge at 3000g → Use sediment to inoculate MGIT same day
- Aliquot C: Untreated → Keep at RT → Add 1%NAOH to equal vol. sample → Use sediment to inoculate MGIT after 8 days
- Aliquot D: Omnigene treated → Add Omnigene and Keep at RT → Centrifuge at 3000g → Use sediment to inoculate MGIT after 8 days

Figure 2: Culture positivity for all aliquots by smear grade
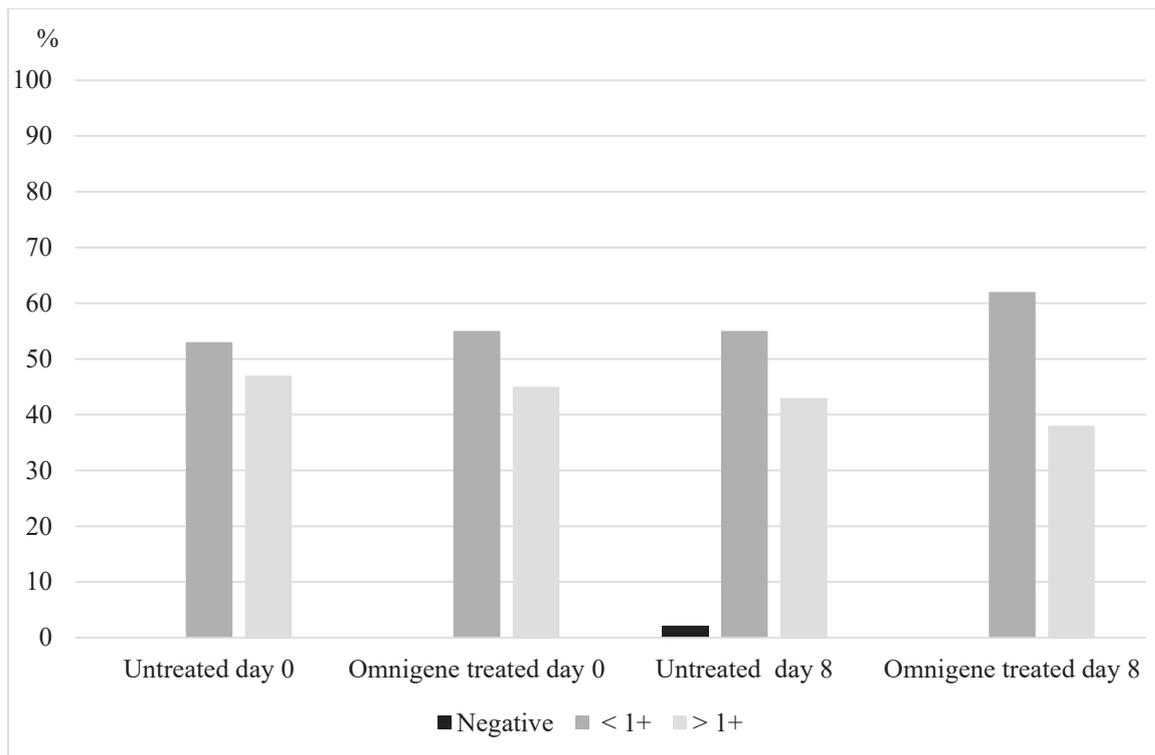
Table 1: Individual Xpert test results

Lab id	Sm pooled sample	UN day 0			UN day 7			UN day 15			OM day 15			ETH day 15		
		Sm	MTB Xpert	RMP Xpert	Sm	MTB Xpert	RMP Xpert	Sm	MTB Xpert	RMP Xpert	Sm	MTB Xpert	RMP Xpert	Sm	MTB Xpert	RMP Xpert
101	3+	3+	high	S	2+	high	S	2+	medium	S	2+	medium	S	2+	high	S
103	1+	1+	low	S	1+	low	S	1+	medium	S	1+	medium	S	1+	low	S
104	1+	2+	medium	S	2+	medium	S	1+	high	S	1+	medium	S	1+	medium	S
105	1+	3AFB	low	S	5AFB	low	S	1AFB	low	S	2AFB	low	S	2AFB	low	S
106	1+	1+	low	S	1+	medium	S	1+	medium	S	1+	medium	S	1+	medium	S
107	1+	1+	high	S	2+	high	S	3+	high	S	2+	high	S	1+	high	S
108	3+	3+	high	S	3+	high	S	3+	high	S	3+	high	S	3+	high	S
109	3AFB	1+	low	S	1+	error	n/a	1+	medium	S	1+	medium	S	1+	medium	S
110	3+	3+	high	S	3+	high	S	3+	high	S	3+	high	S	3+	high	S
111	1+	7AFB	low	S	1+	low	S	1+	low	S	1+	low	S	1+	low	S
112	2+	1+	high	S	1+	medium	S	1+	medium	S	1+	high	S	1+	medium	S
113	1+	1+	medium	S	1+	medium	S	1+	medium	S	1+	medium	S	1+	medium	S
114	2+	2+	medium	S	1+	medium	S	2+	medium	S	2+	medium	S	1+	low	S
115	1AFB	2AFB	low	S	1+	low	S	1AFB	low	S	negative	low	S	negative	negative	n/a
117	1+	10AFB	medium	S	1+	medium	S	1+	medium	S	10AFB	medium	S	11AFB	medium	S
118	3+	2+	medium	S	2+	high	S	2+	high	S	2+	high	S	2+	medium	S
120	1AFB	negative	high	S	negative	low	R	negative	low	R	negative	low	R	1AFB	negative	n/a
121	3+	3+	high	S	3+	high	S	3+	medium	S	3+	high	S	3+	medium	S
122	7AFB	8AFB	medium	S	7AFB	medium	S	negative	medium	S	15AFB	medium	S	12AFB	medium	S
123	15AFB	1+	high	S	2+	high	S	2+	high	S	2+	high	S	2+	medium	S
124	1+	13AFB	medium	S	3AFB	medium	S	2AFB	medium	S	1+	medium	S	2AFB	medium	S
125	2+	15AFB	medium	S	1+	high	S	1+	high	S	12AFB	medium	S	1+	medium	S
126	1+	1+	medium	S	1+	medium	S	1+	medium	S	1+	medium	S	1+	low	S
127	2+	1+	medium	S	2+	high	S	2+	high	S	2+	medium	S	2+	medium	S
128	3+	2+	medium	S	3+	medium	S	3+	medium	S	2+	medium	S	2+	medium	S

130	2+	2+	medium	S	1+	medium	S	2+	medium	S	2+	medium	S	2+	medium	S
131	3+	3+	high	S	3+	high	S	3+	high	S	3+	high	S	3+	high	S
132	3+	3+	high	S	3+	high	S	3+	high	S	3+	high	S	3+	high	S
133	3+	3+	high	S	3+	medium	S	2+	medium	S	3+	high	S	3+	medium	S
134	2AFB	3AFB	low	S	1AFB	very low	S	2AFB	very low	S	3AFB	very low	S	1AFB	very low	S
135	2+	3+	high	S	2+	high	S	2+	medium	S	2+	medium	S	2+	medium	S
136	scanty	8AFB	medium	S	3AFB	medium	S	5AFB	medium	S	5AFB	low	S	2AFB	medium	S
137	1+	2+	medium	S	1+	low	S	1+	medium	S	1+	medium	S	1+	medium	S
138	2+	3+	medium	S	2+	medium	S	3+	high	S	3+	high	S	3+	high	S
139	3+	3+	high	S	3+	high	S	3+	medium	S	3+	high	S	3+	medium	S
140	2+	3+	medium	S	1+	medium	S	3+	high	S	2+	medium	S	3+	medium	S
142	2+	1+	medium	S	2+	high	S	2+	high	S	2+	high	S	2+	very low	S
143	3AFB	1AFB	low	S	6AFB	medium	S	5AFB	medium	S	2AFB	medium	S	2AFB	low	S
144	2+	2+	high	S	1+	error	n/a	3+	high	S	3+	high	S	3+	low	S
145	3+	2+	medium	S	1+	medium	S	2+	high	S	3+	medium	S	3+	medium	S
147	1+	1+	high	S	1+	medium	S	2+	high	S	1+	high	S	1+	medium	S
149	3+	2+	medium	S	2+	high	S	1+	high	S	2+	high	S	2+	high	S
150	1+	1+	high	S	1+	medium	S	1+	medium	S	1+	medium	S	1+	medium	S
151	3+	3+	high	S	3+	high	S	3+	high	S	3+	high	S	3+	high	S
152	3+	2+	medium	S	3+	high	S	3+	low	S	3+	high	S	3+	high	S
153	scanty	2AFB	very low	S	2AFB	low	S	negative	medium	S	negative	medium	S	negative	high	S
154	scanty	2AFB	medium	S	1+	medium	S	5AFB	low	S	1+	low	S	1+	medium	S
155	1+	4AFB	medium	S	7AFB	medium	S	5AFB	low	S	1AFB	medium	S	8AFB	medium	S

MTB: *Mycobacterium tuberculosis*, AFB; Acid Fast Bacilli, UN; Untreated sample, ETH; Ethanol treated sample, sm; smear microscopy results

Table 2: Correlation between Xpert and smear grade for all samples

	≤1+					>1+				
	UND0 n %	UND7 n %	UND15 n %	OMD15 n %	ETHD15 n %	UND0 n %	UND7 n %	UND15 n %	OMD15 n %	ETHD15 n %
Very low /Low	9 (34.6)	8 (28.6)	7 (29.2)	9 (34.6)	8 (28.6)	0	0	1 (4.2)	0	2 (9.1)
Medium/High	17 (65)	18 (64.3)	17 (70.8)	17 (65.4)	18 (64.3)	22 (100)	20 (100)	23 (95.8)	24 (100)	20 (90.9)
Negative/Invalid	0	2 (7.1)	0	0	2 (7.1)	0	0	0	0	0
Total	26	28	24	26	28	22	20	24	24	22

UND0, UND7 and UND15: aliquot untreated tested at day 0, 7, 15 respectively; OMD15: aliquot treated with OM-S tested at day 15; ETH15: aliquot treated with ethanol tested at day 15.

Table 3: Comparison of Xpert results in untreated samples at D0, D7, and D15

		UND7						UND15						Total
		Neg	Very low	Low	Med	High	N/A	Neg	Very low	Low	Med	High	N/A	
UND0	Neg	0	0	0	0	0	0	0	0	0	0	0	0	0
	Very low	0	0	1	0	0	0	0	0	0	1[(1)]	0	0	1
	Low	0	1	4	2	0	1	0	1	3	4	0	0	8
	Med	0	0	1	15	6	0	0	0	3	10	9	0	22
	High	0	0	1[(2)]	4	11	1	0	0	1[(2)]	7	9	0	17
	Invalid	0	0	0	0	0	0	0	0	0	0	0	0	0
	Total	0	1	7	21	17	2	0	1	7	22	18	0	48

1= ID153, 2= ID120

Table 4: Comparison of Xpert results for OM-S and Ethanol treated aliquots at different days

	Results	UND0					UND15					Total
		Neg	Very low	Low	Med	High	Neg	Very low	Low	Med	High	
OMD15	Neg	0	0	0	0	0	0	0	0	0	0	0
	Very low	0	0	1	0	0	0	1	0	0	0	1
	Low	0	0	3	2	1[1]	0	0	5	1	0	6
	Med	0	1[2]	4	15	3	0	0	1	17	5	23
	High	0	0	0	5	13	0	0	1[3]	4	13	18
	Invalid	0	0	0	0	0	0	0	0	0	0	0
	Total	0	1	8	22	17	0	1	7	22	18	48
	Results	UND0					UND15					
		Neg	Very low	Low	Med	High	Neg	Very low	Low	Med	High	Total
ETHD15	Neg	0	0	1	0	1[2]	0	0	2	0	0	2
	Very low	0	0	1	1[3]	0	0	1	0	0	1[3]	2
	Low	0	0	4	2	1[4]	0	0	2	4	1[4]	7
	Med	0	0	2	16	8	0	0	2	16	8	26
	High	0	1[2]	0	3	7	0	0	1[3]	2	8	11
	Invalid	0	0	0	0	0	0	0	0	0	0	0
	Total	0	1	8	22	17	0	1	7	22	18	48

1= ID120, 2= ID153, 3= ID152, 4=ID 142, 5=ID 144

Table 5: Comparison of Xpert results for ETHD15 vs OMD15

	Results	OMD15					Total
		Neg	Very low	Low	Medium	High	
ETHD15	Neg	0	0	2[1]	0	0	2
	Very low	0	1	0	0	1[2]	2
	Low	0	0	2	4	1[3]	7
	Medium	0	0	2	17	7	26
	High	0	0	0	2	9	11
	Invalid	0	0	0	0	0	0
	Total	0	1	6	23	18	48

1= ID115 and ID120, 2= ID142, 3= ID144,

Table 6: Individual culture test results

Lab N°	Sm pooled sample	UN Day 0		OM Day 1		UN Day 8		OM day 8	
		Sm	Culture	Sm	Culture	Sm	Culture	Sm	Culture
202	3+	2+	MTB	2+	MTB	2+	MTB	2+	Negative
203	2+	2+	MTB	3+	MTB	2+	MTB	2+	MTB
204	3+	3+	MTB	3+	MTB	3+	MTB	3+	Negative
205	1+	1+	MTB	1+	Negative	1+	MTB	1+	Negative
206	2+	3+	MTB	3+	Negative	2+	MTB	1+	MTB
207	1+	1+	MTB	1+	MTB	1+	MTB	1+	MTB
208	3+	1+	MTB	3+	MTB	3+	MTB	3+	Negative
209	scanty	1+	MTB	1+	MTB	scanty	MTB	1+	MTB
210	3+	3+	MTB	3+	MTB	3+	MTB	3+	MTB
211	2+	3+	MTB	2+	Negative	2+	MTB	2+	MTB
214	3+	2+	MTB	2+	MTB	3+	MTB	3+	MTB
215	3+	3+	MTB	3+	MTB	3+	MTB	3+	MTB
216	2+	2+	MTB	2+	MTB	2+	MTB	2+	Negative
217	1+	2+	MTB	1+	MTB	1+	MTB	1+	Negative
218	1+	1+	MTB	1+	Negative	1+	MTB	1+	Negative
219	1+	2+	MTB	1+	MTB	1+	MTB	1+	Negative
220	2+	1+	MTB	1+	Negative	1+	MTB	1+	MTB
221	1+	1+	MTB	1+	Negative	1+	MTB	1+	Negative
223	3+	3+	MTB	3+	Negative	3+	MTB	3+	NTM
224	1+	1+	MTB	1+	MTB	1+	MTB	1+	Negative
225	1+	1+	MTB	1+	Negative	1+	MTB	1+	MTB
226	2+	2+	MTB	2+	MTB	1+	MTB	1+	Negative
227	scanty	scanty	MTB	1+	Negative	1+	MTB	1+	Negative
228	1+	1+	Negative	2+	Negative	1+	MTB	1+	MTB
229	3+	3+	Negative	3+	Negative	3+	MTB	2+	MTB
230	2+	2+	MTB	1+	MTB	3+	MTB	2+	MTB
234	1+	1+	MTB	1+	Negative	1+	MTB	1+	Negative
235	1+	1+	MTB	scanty	Negative	1+	MTB	1+	MTB
236	3+	1+	MTB	3+	Negative	3+	MTB	3+	MTB
237	3+	1+	MTB	3+	MTB	3+	MTB	3+	MTB
240	1+	1+	MTB	1+	MTB	1+	NTM	1+	Negative
241	scanty	scanty	MTB	scanty	MTB	scanty	MTB	scanty	Negative
242	scanty	Negative	Contaminated	Negative	Negative	Negative		Not done	Negative
243	1+	1+	MTB	2+	Negative	1+	MTB	1+	Negative
244	2+	2+	MTB	2+	Negative	2+	MTB	2+	MTB
245	2+	2+	MTB	1+	Negative	1+	MTB	1+	Negative

246	3+	3+	MTB	3+	Negative	3+	MTB	3+	Negative
248	3+	3+	MTB	3+	Negative	3+	MTB	3+	Negative
249	3+	2+	MTB	3+	MTB	3+	MTB	3+	MTB
250	scanty	scanty	MTB	scanty	MTB	1+	MTB	1+	MTB
252	1+	1+	Negative	1+	Negative	1+	MTB	1+	Negative
253	3+	3+	MTB	3+	Negative	3+	MTB	3+	Negative
254	1+	1+	MTB	1+	Negative	1+	MTB	1+	Negative
255	1+	1+	MTB	1+	Negative	1+	Negative	1+	Negative
256	3+	2+	Negative	1+	Negative	2+	MTB	1+	Negative
257	1+	scanty	Negative	scanty	Negative	scanty	MTB	scanty	Negative
260	1+	1+	MTB	1+	MTB	1+	MTB	1+	MTB

MTB; *Mycobacterium tuberculosis*, UN; Untreated sample, OM; Omnigene treated sample, Sm; smear microscopy results

Table 7: Comparison of culture results of OMD8 with UND0, UND8 and OMD0 samples

		UND0				UND8				OMD0		
		Neg	MTB	NTM	Cont	Neg	MTB	NTM	ND	Neg	MTB	NTM
OMD8	Neg	3	22	0	1	1	23	1	1	16	10	0
	MTB	2	18	0	0	0	20	0	0	9	11	0
	NTM	0	1	0	0	0	1	0	0	1	0	0
	Total	5	41	0	1	1	44	1	1	26	21	0

Cont.: culture contaminated; Neg: culture negative; NTM: *non-tuberculous mycobacteria:* UND0 and UND88: aliquot untreated tested at day 0 and day 8; OMD0: aliquot treated with OM-S tested at day 0;

Table 8: Comparison of culture results of UND0 with UND8 and OMD0 samples

		UND8				OMD0		
		Neg	MTB	NTM	ND	Neg	MTB	NTM
UND0	Neg	0	5	0	0	5	0	0
	MTB	1	39	1	0	20	21	0
	Cont	0	0	0	1	1	0	0
	Total	1	44	1	1	26	21	0

ND: not done; Cont.: culture contaminated; Neg: culture negative; NTM: *non-tuberculous mycobacteria:* UND0 and UND8: aliquot untreated tested at day 0 and day 8; OMD0: aliquot treated with OM-S tested at day 0

Evaluation of the Omnigene reagent for long term preservation of sputum for MGIT culture in Uganda

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Abstract

Background: Mycobacteria Growth Indicator Tube (MGIT) culture is highly sensitive for isolation of TB from sputum and is mostly implemented in central or reference laboratories. To reach these facilities samples often require long transport time at room temperature (RT), resulting in increased contamination and loss of mycobacteria viability. Omnigene (OM) is a reagent to preserve sputum up to 8 days without cold chain but requires further evaluation.

Design/Methods: Fifty smear-positive newly-diagnosed patients from Uganda submitted 2 to 3 sputum samples that were pooled to achieve 10ml final volume. Four aliquots were prepared and tested with microscopy to check for homogeneity. Two of the aliquots were inoculated in MGIT at date of collection (D0); one added with Omnigene and one after decontamination with 1% sodium hydroxide (NaOH). The remaining two aliquots were inoculated after 8 days (D8) of storage at room temperature, and treated as above; with Omnigene and 1% NaOH respectively. MTB culture yield and rate of contamination were compared between Omnigene and NaOH treated aliquots at each time point.

Results: Of 200 aliquots, 46% were ≥ 2+ by microscopy: 24/50 (48%) and 21/49 (43%) among Omnigene treated aliquots at D0 and D8, and 24/50 (48%) and 23/50 (46%) among NaOH treated aliquots respectively.

After exclusion of contaminated and NTM, Mycobacteria tuberculosis (MTB) was isolated in 21/50 (42%) and 21/49 (43%) Omnigene treated aliquots and 43/49 (88%) and 46/49 (94%) NaOH

treated aliquots at D0 and D8 respectively. Only 1 (2%) contaminated and 1 (2%) NTM cultures were observed among NaOH treated aliquots at D0 and D8 respectively.

Conclusions: In this proof of concept study, we observed an important loss of MTB recovery by MGIT among the Omnigene treated aliquots. Optimization of the Omnigene reagent is needed before its use to preserve samples before MGIT culture.

Evaluation of the OMNIgene® SPUTUM reagent for long term transportation of samples for Xpert testing in a high TB-HIV setting

epicentre

MEDECINS SANS FRONTIERES

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Background and objective

- Xpert MTB/RIF has greatly improved tuberculosis diagnosis.
- The use of Xpert is limited by the need of electricity, supplies and maintenance costs.
- As a result, in many settings samples still require long transportation to reference laboratories to be tested.
- Currently, Xpert testing is recommended within 7 days from sample collection.
- OMNIgene® (DNA Genotek, Canada) is a new reagent for sample preservation.
- Ethanol is a known preservative for DNA but with limited evidence for Xpert MTB/RIF testing
- This laboratory proof of concept study assessed the Xpert detection yield for samples stored in ethanol, Omnigene and preservative-free up to 15 days at room temperature

Methods

Study site: Mbarara, South West Uganda.

Study population
- Newly diagnosed smear positive pulmonary adults
- Informed consent to participate

Sample size
50 consecutive patients

Laboratory procedures
- Two to three Smear positive samples (min volume of 5ml) were pooled together
- Samples were gently vortexed and split into five aliquots (Fig 1), checked for microscopy homogeneity
- Untreated sputum aliquots were tested with Xpert MTB/RIF at day 0, 7 and 15, with OMNIgene® and ethanol at day 15 (Figure 1)
- Xpert MTB/RIF testing followed manufacturer's guidelines

Figure 1: Sample flow chart
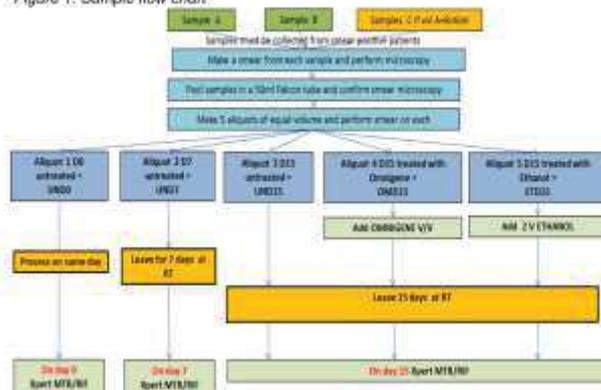
References: Cepheid. Operator Manual test Xpert MTB / RIF. 2009.

Results

- 55 participants screened and 50 enrolled
  5 excluded because of smear negativity
- Majority of participants 70% (35/50) were males
- 250 aliquots tested with smear-microscopy
  - Smear negative: 18 (7.2%)
  - Smear positive scanty: 53 (21.2%)
  - Smear positive 1+: 67 (26.8%)
  - Smear positive 2+: 52 (20.8%)
  - Smear positive 3+: 60 (24%)
- Xpert MTB/RIF results of the 250 aliquots globally (Table 1)
  4 (1.6%) invalid results (not re-tested)

Table 1: Xpert results by smear grade

Xpert results	Negative, scanty, 1+ (n=138 aliquots)					≥ 2+ (n= 112 aliquots)				
	UND0 N=28	UND7 N=30	UND15 N=26	OMD15 N=28	ETD15 N=28	UND0 N=22	UND7 N=20	UND15 N=24	OMD15 N=24	ETD15 N=22
N, Pos	28	28	26	26	26	22	20	24	24	22
High	6 (21.4)	1 (3.6)	3 (11.5)	2 (7.7)	2 (7.7)	11 (50)	16 (80)	15 (62.5)	16 (66.7)	9 (40.9)
Medium	11 (39.3)	18 (64.3)	15 (57.7)	14 (53.8)	15 (57.7)	11 (50)	4 (20)	8 (33.3)	8 (33.3)	11 (50)
Low	10 (35.7)	8 (28.6)	6 (23.1)	7 (26.9)	7 (26.9)	0	0	1 (4.2)	0	1 (4.5)
Very Low	1 (3.6)	1 (3.6)	2 (7.7)	3 (11.3)	2 (7.7)	0	0	0	0	1 (4.5)
N, Neg	0	0	0	0	0	0	0	0	0	0
N, Invalid	0	2	0	0	2	0	0	0	0	0

- At day 15, 40/50 (80%) of aliquots treated with OMNIgene® and 37/50 (74%) with Ethanol had Xpert TB detected with high or medium grade vs 41/50 (82%) for untreated aliquots, p=0.92.
- Low smear grades (<2+), at day 15; Xpert detection was high or medium grade in 16/26 (61.5%) and 17/26 (65.4%) for OMNIgene® and ethanol treated aliquots vs 18/26 (69.2%) for untreated, p=0.91 and p=0.68.

Conclusion

- OMNIgene® did not improve TB detection in aliquots preserved up to 15 days.
- No difference with the delay in time for samples without preservatives.
- OMNIgene® did not add value to untreated sample; no significant difference in detection.
- Ethanol did not add value to untreated sample as well.
- Additional evaluation could be explored with a large number of smear-negative samples and also for aliquots stored beyond 15 days (mainly for surveys).

Chapter 4

GENERAL DISCUSSION

4.1 Diagnosis of childhood TB

In this work, we show further evidence in laboratory diagnosis of pediatric TB and highlight the existing challenges especially in our resource-limited setting. In particular, we documented the potential impact of poor diagnostic tools on the outcome of children identified with presumptive TB in high burden and limited resource setting. Indeed, mortality of children started on empirical TB treatment was much higher than the one of children treated with microbiologically confirmed TB. As discussed earlier, this can just highlight the likelihood of initiating empirical treatment in children admitted with very poor conditions but it could also be explained by misdiagnosis of other affections especially in children with comorbidities such as HIV infection or severe malnutrition. These results highlight the urgent need of better diagnostic tools for children, preferably not relying on sputum or respiratory specimen.

This was among the pioneer studies to explore testing Xpert on stool samples and obtained promising results. Since this study, more evidence has shown that stool can be used to diagnose TB in children although the sensitivity remains lower than that of Xpert from respiratory samples (120,161,187–189). However, the lower sensitivity of Xpert in stool as compared to gastric aspirate or induced sputum is likely to be compensated by a much higher proportion of children accessing stool collection methods compared to classical sputum alternatives requiring equipment, safety measures, inpatient facility that cannot be easily provided in facilities of limited resource settings. Some studies have highlighted the very low diagnostic capacity of pediatric TB in facilities of resource-limited settings (190,191). Therefore, under routine conditions collecting stool in children unable to provide sputum for Xpert testing could in the end detect more children than would be missed by more demanding methods. Hence this needs to be further documented in programmatic conditions.

However, the need to process the stool specimen in order to remove big particles that can inhibit the PCR reaction, which often requires specimen manipulation and specimen centrifugation remains an operational challenge for the use of Xpert from stool in many facilities of limited resource countries. This explains also the heterogeneity between the different stool specimen processing methods as reported in the recent systematic review and could be a limitation for a rapid endorsement of the method by WHO(122).

Further optimization of stool processing in order to increase the detection yield but also to reduce on the infrastructural steps such as centrifugation that require electricity, vortexing, safety cabinets, and several laboratory supplies, advanced training that are not readily available in lower settings is needed. Some studies have reported promising results using sedimentation method instead of centrifugation (192). Other have reported very promising results using very simple approach that is under evaluation (STEP methods) and FIND is supporting the development of stool processing kits that could also simplify the processing steps and allow its use at primary health care setting but is still under evaluation (177,189,193).

Our study was performed before the release of the new Xpert cartridge, Ultra that has a higher detection of up to 16cfu and can increase the detection yield from respiratory samples in children (194,195). By using Ultra technology in stool it can help to increase sensitivity but also reduce turnaround time, hence providing quick treatment initiation and avoid over waiting.

One other possibility is to increase detection yield of existing tests by using a combined strategy of non-sputum samples. This indicates the importance of collecting and testing for more than one sample especially the well tolerated NPA sample and easily available stool.

This study was one of the few studies evaluating the performance of the urine LAM test for diagnosis of TB in children. In this study, we deliberately decided to focus on children with poor prognosis either due to critical presentation at admission or high risk of disseminated TB. This was justified by the urgency to improve diagnosis of children with severe illness as highlighted by the results of the analysis of outcomes from our first pediatric cohort study and by the hypothesis that the urine LAM could be able to identify these children based on previous tests results in advanced HIV infected adults. We found a fair sensitivity similar to what was reported in studies in adults and children but a poor specificity. In adults, the urine LAM has a high specificity and studies in children have reported discordant results regarding the specificity(100,161,178). The loss of specificity might be due to the increased risk of sample contamination in children as compared to adults. Although the manufacturer of the kit indicates that the test can only detect arabinomanan proteins that are specific for TB. This assertion requires further investigation. More recently, this protein has also been reported in HIV infected adults with disseminated NTM infections(196) implying that these mycobacteria may affect the interpretation of the test.

In both studies enrolling children with presumptive TB, we observed that the proportion of bacteriological confirmed TB was much lower than expected despite exhaustive sample collection and use of both Xpert and mycobacterial culture methods for TB testing. This is not very consistent with the recent national TB prevalence survey reporting higher prevalence of the TB in the general population of Uganda than what was estimated by WHO based on TB notification reports from the national TB control program(30). This was particularly surprising in the second cohort of children with severe illness. Although Xpert and culture from respiratory samples are imperfect reference standards for diagnosis of TB in children, the relatively low proportion of children classified as confirmed or unconfirmed TB using the uniform case definitions of intrathoracic TB was also in favor of a low prevalence of TB in this study population. This leaves some unanswered yet important questions regarding the actual prevalence of TB in children attending the Mbarara Regional Referral Hospital and necessitates further exploration. One assumption is that symptomatic children might not be screened but referred for specialized care to private or private not for profit health institutions for advanced care.

4.2 Sample preservation for TB testing

In resource-limited settings, there is need to transport samples from the peripheral health facility to reference laboratories in order to access Xpert and culture technology. Sample delay before processing may affect the performance of the tests. The Global Laboratory Initiative guidelines recommend sample referral systems to help prevent the need for patients to travel and provide a more equity access to TB diagnosis and care. In Uganda, the national TB reference laboratory through public-private partnership established a strong referral system using a comprehensive and trained team via the postal system (197). In a nutshell, the government of Uganda uses a hub system where motorcycle riders collect all referral samples from various health facilities and assemble them at a regional hub with capacity to perform Xpert testing. At the same time, samples for culture and DST are packaged at the regional hub and via post office to national reference Lab where subsequently the tests are performed.

The use of a simple reagent that can preserve the viability of TB longer than the recommended time for culture or Xpert without cold chain would facilitate the system of sample transport and improve diagnostic capacity even in countries with a hub system like in Uganda. Although our study was exploratory, we showed that addition of Omnigene or ethanol to a smear-positive sputum does not affect the performance of Xpert after a period of 2 weeks of storage at room temperature. These encouraging results were balanced by the fact that similar results were obtained with samples stored for 2 weeks in same conditions without preservative. However, the storage conditions of sample in a BSL3 laboratory (at 22-26°C) are expected to be quite different to the storage conditions in a peripheral laboratory and further assessment in such conditions are required. On the other hand, others did report increase of Xpert detection with Omnigene as shown in previous studies (142,146).

The study shows that treatment of sputum with Omnigene caused significant loss of viability of bacilli when using MGIT culture. Our findings are in line with those reported by other investigators(144,145).

We recommend that in the current conditions, it is possible to transport samples without cold chain from the field for Xpert testing and culture but test these within two weeks and one week respectively. The use of fresh samples however remains the best option in settings where the technology is available.

4.3 Perspectives of paediatric TB diagnosis

Improving childhood TB diagnosis requires several strategies in combination. Funded by UNITAID, Uganda with Epicentre is among the 7 countries participating in an international multicenter study entitled "Strengthening paediatric TB services for enhanced early detection" shorted as TB Speed. The aim of this project is to improve TB case detection using several innovative approaches including the decentralization of pediatric TB diagnosis at district hospital and primary health centres levels; the systematic TB detection among children admitted with

severe pneumonia and the improvement of diagnostic algorithms of children with comorbidities such as severe acute malnutrition and HIV infection. The microbiology component of the study is focusing at child-friendly sample collection methods using nasopharyngeal aspirate and stool, and use of Xpert Ultra testing displayed in peripheral laboratories using optimized GeneXpert technology (GeneXpert Edge). The diagnostic approach also includes training and mentoring of clinicians and nursing for clinical and radiological diagnosis of TB and use of digitalized X-ray to enhance quality and facilitate quality control.

The TB-Speed project is also aiming at improving the Xpert from stool with the optimization of the stool processing in order to allow its use at low level of health care facility. Epicentre is associated with a head to head study comparing three promising stool specimen processing methods that are all centrifuge free among children with presumptive TB in Uganda and Zambia: a sucrose-flotation method, the One Step method based on the simple dilution of stool before Xpert testing and the Stool Processing Kit using stool concentration after dilution with a stool processing buffer (196,(120).

Finally, Epicentre is also associated with a multicentric evaluation of the performance of the new urine LAM test (Fuji LAM) among different population of HIV infected adults and children with support from FIND.

These progresses should not discourage the research of biomarkers to obtain a non-sputum based point of care test, such as the IP10 that has been identified as a potential interesting inflammatory biomarker for diagnosis of TB in children (198); the blood Monocyte-to-Lymphocyte ratio (MLR)(198–200), and host metabolic response to MTB infection(201).

SUMMARY

The world health organization estimates that in 2017, close to 1 million children below 15 years developed tuberculosis but only half of them were notified. Difficulty to obtain sputum in children and the paucibacillary nature of intrathoracic childhood tuberculosis challenge the diagnosis of tuberculosis in children. This leads to the common use of empirical treatment with a high risk of over or under diagnosis. Besides that, few facilities in low resource settings have adequate laboratory capacity to diagnose tuberculosis. Samples must be transported to a reference laboratory, which can effect performance of the tests, especially in the absence of cold chain.

Three studies were conducted in Mbarara (Uganda) to evaluate non-respiratory samples and specimen preservation methods to improve diagnosis of pediatric tuberculosis. In the first study, we assessed the performance of Xpert MTB/RIF on sputum and stool in children with presumptive tuberculosis and documented outcomes of children according to the tuberculosis treatment decision. In the second study, we assessed the performance of stool Xpert MTB/RIF and urine lipoarabinomanann (LAM) among children admitted with severe illness. In the 3rd study, we determined Xpert MTB/RIF and MGIT culture recovery rates of smear positive sputum specimen kept untreated at room temperature and treated with either Omnigene or ethanol over different time periods.

Of 392 children (median age 3.9 years, 45.4% female and 31% HIV infected) enrolled in the 1st study, 4.3% (17/392) were microbiologically confirmed tuberculosis. Using a microbiological reference standard, sputum Xpert MTB/RIF had a 90.9% sensitivity and specificity of 99.1%. The sensitivity and specificity of stool Xpert MTB/RIF was 55.6% and 98.2%. The study reported mortality of 6.9% within three months with a higher proportion (10.7%) among children treated for tuberculosis compared to the non-treated children (4.5%). None of treated children with bacteriologically confirmed tuberculosis died compared to 12.3% of those treated empirically.

Of 234 patients (median age 16.5 months, 48.3% female, 31.6% HIV infected, 58.5% severely malnourished) enrolled in the 2nd study, 5.1% were microbiologically confirmed tuberculosis. Stool Xpert MTB/RIF had a sensitivity of 50% and specificity of 99.1%. For the urine LAM test, it was 50% and 74.1%, respectively. False positive LAM results were more common among low grade positive LAM results and occurred more frequently when urine samples had bacterial contamination.

The 3rd study documented that by 15th day, there was no difference of Xpert MTB/RIF recovery rate between samples treated with Omnigene or ethanol and untreated samples, meaning that in the study conditions there was no benefit of adding any preservative for samples stored at room temperature up to 15 days. We observed a substantial loss of viability of *Mycobacterium tuberculosis* on samples treated with Omnigene, which does not support the use of Omnigene for sample transportation before MGIT testing.

In conclusion, Xpert MTB/RIF on stool gave promising results for the use in children unable to provide sputum and could be an interesting alternative to more complex methods such as sputum induction and gastric aspirate for primary health care centers of limited resource countries. The low specificity of the urine LAM requires further investigation before its use for diagnosis of tuberculosis in children. Despite the encouraging Xpert MTB/RIF results from specimen preserved either with Omnigene or ethanol further evaluation under routine field conditions is necessary.

Short summary

Diagnosis of tuberculosis is challenged by the difficulty to obtain sputum in children. Therefore, most children are started on treatment empirically. In low resource settings most health facilities lack capacity to perform laboratory diagnosis, requiring sample transportation to reference laboratories, which can affect test performance. In three studies in Uganda, we evaluated performance of the molecular assay Xpert MTB/RIF on stool and urine LAM test in children with presumptive tuberculosis and preservation techniques to delay testing of sputum sample stored without cold chain.

Although less performant than sputum, stool Xpert could be a good alternative for children unable to produce sputum. On the other hand, many false positive urine LAM results questions its use. Omnigene and ethanol were able to maintain the same level of Xpert detection after 2 weeks of storage.

Résumé

L'Organisation Mondiale de la Santé estime qu'en 2017 près d'un million d'enfants de moins de 15 ans ont développé la tuberculose mais seulement la moitié des cas ont été notifiés. Les difficultés pour recueillir des échantillons de crachat chez les enfants et la nature paucibacillifère de la tuberculose pédiatrique représentent de véritables challenges diagnostiques. Cela aboutit à la prescription fréquente de traitement empirique avec un risque de sur- ou sous-diagnostic. De plus, peu de laboratoires dans les pays à ressources limitées ont les capacités du diagnostic de la tuberculose. Les échantillons doivent être transportés vers des laboratoires de référence pouvant affecter les performances des tests, notamment en l'absence de chaine de froid.

Trois études ont été menées à Mbarara (Ouganda) pour évaluer des échantillons non-respiratoires et des méthodes de conservation des échantillons pour améliorer le diagnostic de la tuberculose de l'enfant. Dans la première étude, nous avons évalué les performances de l'Xpert MTB/RIF sur les expectorations et les selles d'enfants avec présomption de tuberculose et nous avons documenté le devenir des enfants selon la décision thérapeutique. Dans la deuxième étude, nous avons évalué les performances de l'Xpert MTB/RIF dans les selles et du test lipoarabinomanann (LAM) dans les urines chez des enfants admis dans un état critique. Dans la troisième étude, nous avons déterminé le taux de détection avec Xpert MTB/RIF et la culture MGIT d'échantillons de crachats frottis-positifs conservés à température ambiante sans traitement, ou traités avec Omnigène ou éthanol à différents périodes de temps.

Sur 392 enfants (âge médian 3,9 ans, 45,5% de filles et 31% VIH positifs) inclus dans la 1e étude, 4,3% ont été confirmés microbiologiquement. L'Xpert MTB/RIF dans le crachat avait une sensibilité de 90,9% et une spécificité de 99,1% contre un test de référence microbiologique. La sensibilité et la spécificité de l'Xpert dans les selles étaient de 55,6% et 98,2%. La mortalité était de 6,9% à trois mois, et était plus importante chez les enfants traités (10,7%) que chez les enfants non-traités (4,5%). Aucun des enfants traités pour une tuberculose microbiologiquement confirmée n'est décédé contre 12,3% de ceux traités de façon empirique.

Parmi les 234 enfants (âge médian 16,5 mois, 48,3% de filles, 31,6% VIH positifs et 58,5% sévèrement malnutris) inclus dans la 2e étude, 5,1% avaient une tuberculose microbiologiquement confirmée. Xpert MTB/RIF dans les selles avait une sensibilité de 50% et une spécificité de 99,1%.

La sensibilité du test urinaire LAM était de 50% et la spécificité de 74,1%. Les faux positifs LAM étaient plus fréquents parmi les résultats positifs LAM de bas grade et dans les urines avec une contamination bactérienne.

Dans la 3^e étude, après 15jours, il n'y avait pas de différence de détection par Xpert MTB/RIF entre les échantillons traités avec Omnigène ou éthanol et les échantillons non traités, ne montrant pas de bénéfice de l'ajout d'un conservateur. Nous avons décrit une baisse substantielle de viabilité de *Mycobacterium tuberculosis* dans les échantillons traités par Omnigène, ce qui n'est pas en faveur de l'utilisation de l'Omnigène pour le transport des échantillons avant culture MGIT.

En conclusion, Xpert MTB/RIF dans les selles a montré des résultats prometteurs chez les enfants ne pouvant pas cracher et pourrait être une alternative intéressante à des méthodes plus complexes comme l'induction du crachat et l'aspiration gastrique pour les centres de santé primaire des pays à ressources limitées. La faible spécificité du LAM dans les urines nécessite des investigations complémentaires avant son utilisation pour le diagnostic de la tuberculose de l'enfant. En dépit des résultats encourageants de l'Xpert MTB/RIF sur les échantillons conservés avec Omnigène ou l'éthanol, des investigations complémentaires dans des conditions programmatiques sont nécessaires.

Résumé court

Le diagnostic de la tuberculose est contrait par la difficulté des enfants à produire des expectorations. En conséquence, la plupart des enfants sont traités de façon empirique. Dans les contextes à ressources limitées, les laboratoires n'ont pas tous les capacités diagnostiques de la tuberculose nécessitant le transport des échantillons vers un laboratoire de référence affectant les performances des tests.

Dans trois études en Ouganda, nous avons évalué les performances du test moléculaire Xpert MTB/RIF sur selles et test antigénique urinaire LAM chez des enfants avec présomption de tuberculose et des méthodes de conservation d'échantillons sans chaine de froid.

Bien que moins performant que sur les expectorations, l'Xpert sur selles semble une bonne alternative pour les enfants ne pouvant pas cracher. Le nombre important de faux positifs LAM ne questionnent sa recommandation. L'Omnigene et l'éthanol ont permis de maintenir le même taux de détection Xpert après 2 semaines de stockage.

REFERENCES

1. Pfyffer GE. Mycobacterium: General Characteristics, Laboratory Detection, and Staining Procedures*. In: Manual of Clinical Microbiology, 11th Edition. 2015.

2. Kubica G, Kent K. Public health mycobacteriology: a guide for the level III laboratory. Atlanta: Department of Health and Human\rServices, Public Health Service, Centers for Disease Control. 1985.

3. Joint Tuberculosis Committee of the BTS S of the. Management of opportunist mycobacterial infections: Joint Tuberculosis Committee Guidelines 1999. Subcommittee of the Joint Tuberculosis Committee of the British Thoracic Society. Thorax. 2000;

4. Wagner D, Young LS. Nontuberculous mycobacterial infections: A clinical review. Vol. 32, Infection. 2004. p. 257–70.

5. Koh WJ, Kwon OJ, Lee KS. Nontuberculous Mycobacterial Pulmonary Diseases in Immunocompetent Patients. Korean Journal of Radiology. 2002.

6. WHO. WHO Global Tuberculosis Report 2018. Reports. 2018.

7. Daniel TM. The history of tuberculosis. Respir Med. 2006;

8. WHO. Global Tuberculosis Report 2018. Vol. 69, Pharmacological Reports. 2017. 683-690 p.

9. Centers for Disease Control and Prevention. Basic TB Facts. CDC Factsheet. 2012;

10. Rose CE, Zerbe GO, Lantz SO, Bailey WC. Establishing priority during investigation of tuberculosis contacts. Am Rev Respir Dis. 1980;

11. Almeida LMD, Barbieri MA, Da Paixão AC, Cuevas LE. Use of purified protein derivative to assess the risk of infection in children in close contact with adults with tuberculosis in a population with high Calmette-Guérin bacillus coverage. Pediatr Infect Dis J. 2001;

12. Grzybowski S, Barnett GD, Styblo K. Contacts of cases of active pulmonary tuberculosis. Bull Int Union Tuberc. 1975;

13. Lienhardt C, Sillah J, Fielding K, Donkor S, Manneh K, Warndorff D, et al. Risk factors for tuberculosis infection in children in contact with infectious tuberculosis cases in the

Gambia, West Africa. Pediatrics. 2003;

14. Pai M, Behr MA, Dowdy D, Dheda K, Divangahi M, Boehme CC, et al. Tuberculosis. Nat Rev Dis Prim [Internet]. 2016;2:16076. Available from: http://www.nature.com/articles/nrdp201676

15. Achkar JM, Jenny-Avital ER. Incipient and subclinical tuberculosis: Defining early disease states in the context of host immune response. J Infect Dis. 2011;204(SUPPL. 4):1179–86.

16. Drain PK, Bajema KL, Dowdy D, Dheda K, Naidoo K, Schumacher SG, et al. Incipient and subclinical tuberculosis: A clinical review of early stages and progression of infection. Clin Microbiol Rev. 2018;31(4):1–24.

17. Agizew T, Bachhuber MA, Nyirenda S, Makwaruzi VZSAM, Tedla Z, Tallaksen RJ, et al. Association of chest radiographic abnormalities with tuberculosis disease in asymptomatic HIV-infected adults. Int J Tuberc Lung Dis. 2010;14(3):324–31.

18. World Health Organization. Treatment of tuberculosis: guidelines – 4th ed. World Health Organization, & Stop TB Initiative (World Health Organization). 2010.

19. Cruz AT, Starke JR. Clinical manifestations of tuberculosis in children. Paediatric Respiratory Reviews. 2007.

20. Gedde-dahl T. Tuberculous infection in the light of tuberculin matriculation. Am J Epidemiol. 1952;

21. Marais BJ, Gie RP, Schaaf HS, Hesseling AC, Obihara CC, Starke JJ, et al. The natural history of childhood intra-thoracic tuberculosis: A critical review of literature from the pre-chemotherapy era. International Journal of Tuberculosis and Lung Disease. 2004.

22. WHO. Guidance for national tuberculosis programmes on the management of tuberculosis in children Second edition. Mater Res Bull. 2014;

23. World Health Organization. Rapid Advise: Treatment of tuberculosis in children [Internet]. Geneva Swtzerland,. Geneva, Switzerland; 2010. Available from: WHO/HTM/TB/2010.13.

24. World Health Organisation. Consolidated Guidelines on Tuberculosis Treatment. 2019.

25. Fogel N. Tuberculosis: A disease without boundaries. Tuberculosis. 2015.

26. Orme IM. Tuberculosis vaccine types and timings. Clinical and Vaccine Immunology. 2015.

27. Getahun H, Matteelli A, Chaisson RE RM. Latent Mycobacterium tuberculosis infection. N Engl J Med. 2015;372(22):2127–35.

28. World HealthOrganisation. Latent tuberculosis infection:Updated and consolidated guidelines for programmatic management. 2018.

29. Narasimhan P, Wood J, Macintyre CR, Mathai D. Risk factors for tuberculosis. Pulmonary Medicine. 2013.

30. WHO Uganda. Ministry of Health and partners review the National TB prevalence survey report. 2017.

31. Ministry of Health. Manual for management and control of tuberculosis and leprosy. 2017.

32. WHO. Tuberculosis Country profile: Uganda. Bulletin [Internet]. 2019;(2019-04-23). Available from: www.who.int/tb/data

33. Republic of Uganda. Direct and Indirect costs due to Tuberculosis and proportion of Tuberculosis-a_ected households experiencing catastrophic costs due to TB in Uganda. Kampala; 2019.

34. Global TB report 2017.

35. Global TB report 2016.

36. WHO. Global Tuberculosis Report 2015. Global Tuberculosis Report 2015. 2015.

37. Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, Raviglione MC, et al. The growing burden of tuberculosis: Global trends and interactions with the HIV epidemic. Archives of Internal Medicine. 2003.

38. Graham SM. Desk-guide for diagnosis and management of TB in children. Geneva Swtzerland, 2016. 2016;

39. Dodd PJ, Gardiner E, Coghlan R, Seddon JA. Burden of childhood tuberculosis in 22 high-burden countries: A mathematical modelling study. Lancet Glob Heal. 2014;

40. Dodd PJ, Yuen CM, Sismanidis C, Seddon JA JH. The global burden of tuberculosis mortality in children: a mathematical modelling study. Lancet Glob Heal.

2017;5(9)::e898-e906.

41. Teklay G, Teklu T, Legesse B, Tedla K, Klinkenberg E. Barriers in the implementation of isoniazid preventive therapy for people living with HIV in Northern Ethiopia: A mixed quantitative and qualitative study. BMC Public Health. 2016;

42. Marais BJ. Improving access to tuberculosis preventive therapy and treatment for children. Vol. 56, International Journal of Infectious Diseases. 2017.

43. Dodd PJ, Sismanidis C, Seddon JA. Global burden of drug-resistant tuberculosis in children: a mathematical modelling study. Lancet Infect Dis. 2016;

44. UNICEF. HIV/ AIDS (Global and Regional trends). UNICEF Data: Monitoring the Situation of Children and Women. 2015.

45. Tobin NH, Aldrovandi GM. Immunology Of Pediatric HIV Infection. Immunol Rev. 2013;

46. House L. hiv & aids treatment in practice. (part 1):1–10.

47. World Health Organization. Guideline on When To Start Antiretroviral Therapy and on Pre-Exposure Prophylaxis for HIV. World Health Organization. 2015.

48. Dodd PJ, Prendergast AJ, Beecroft C, Kampmann B, Seddon JA. The impact of HIV and antiretroviral therapy on TB risk in children: A systematic review and meta-analysis. Thorax. 2017;

49. World Health Organisation. World Health Statistics: Monitoring Health for SGDs. 2018.

50. Republic of Uganda. Uganda Population Based HIV impact assesment 2016-2017. Report. 2017;

51. Ministry of Health. Isoniazid Preventive Therapy in Uganda:A Health Worker's Guide. Bulletin. 2014;

52. Ubesie A, Ibeziakor N. High burden of protein-energy malnutrition in Nigeria: Beyond the health care setting. Ann Med Health Sci Res. 2012;

53. UNICEF/WHO/World Bank Group. Levels and trends in child malnutrition 2018. Joint Child Malnutrition Estimates 2018 edition. 2018.

54. UN. The Sustainable Development Goals Report. United Nations Publications. 2018.

55. von Grebmer K, Bernstein J, Patterson F, Sonntag A, Klaus LM, Fahlbusch J, et al. 2018

Global Hunger Index. 2018;68. Available from: https://www.globalhungerindex.org/

56. Karyadi E, Schultink W, Nelwan RHH, Gross R, Amin Z, Dolmans WM V., et al. Poor Micronutrient Status of Active Pulmonary Tuberculosis Patients in Indonesia. J Nutr. 2018;

57. Lönnroth K, Castro KG, Chakaya JM, Chauhan LS, Floyd K, Glaziou P, et al. Tuberculosis control and elimination 2010-50: cure, care, and social development. The Lancet. 2010.

58. Chandra RK. Nutrition and the immune system: An introduction. American Journal of Clinical Nutrition. 1997.

59. Kumar R, Singh J, Joshi K, Singh HP. Hospitalized Children with Severe Acute Malnutrition in Rewa District. Indian Pediatr. 2013;

60. Zachariah R, Spielmann MP, Harries AD, Salaniponi FML. Moderate to severe malnutrition in patients with tuberculosis is a risk factor associated with early death. Trans R Soc Trop Med Hyg. 2002;

61. Hicks RM, Padayatchi N, Shah NS, Wolf A, Werner L, Sunkari VB, et al. Malnutrition associated with unfavorable outcome and death among South African MDR-TB and HIV co-infected children. Int J Tuberc Lung Dis. 2014;

62. Bonnet M, Nansumba M, Bastard M, Orikiriza P, Kyomugasho N, Nansera D, et al. Outcome of children with presumptive tuberculosis in Mbarara, Rural Uganda. Pediatr Infect Dis J. 2018;

63. Wagnew F, Worku W, Dejenu G, Alebel A, Eshetie S. An overview of the case fatality of inpatient severe acute malnutrition in Ethiopia and its association with human immunodeficiency virus/tuberculosis comorbidity-a systematic review and meta-analysis. Int Health. 2018;

64. USAID-DHS. Uganda: Nutrition Profile. 2018;10(April):1–6. Available from: https://www.usaid.gov/sites/default/files/documents/1864/Uganda-Nutrition-Profile-Apr2018-508.pdf

65. Shively G, Hao J. A Review of Agriculture, Food Security and Human Nutrition Issues in Uganda. Purdue Univ West Lafayette, [Internet]. 2012;(September):1–42. Available from:

http://ageconsearch.umn.edu/bitstream/135134/2/12-3Shively.Hao.pdf

66. Bonnet M, Nansumba M, Bastard M, Orikiriza P, Kyomugasho N, Nansera D, et al. Outcome of Children With Presumptive Tuberculosis in Mbarara, Rural Uganda. Pediatr Infect Dis J. 2018;

67. World Health Organization. Global strategy and targets for tuberculosis prevention, care and control after 2015. Who. 2014.

68. Plan G, Tb E. THE PARADIGM SHIFT 2016-2020. 2016;

69. Organization. WH. Compendium of WHO guidelines and associated standards: ensuring optimum delivery of the cascade of care for patients with tuberculosis. ocument WHO/HTM/TB/201713 Geneva, World Heal Organ 2017. 2017;

70. World Health Organization. Xpert MTB / RIF implementation Manual. 2014.

71. World HealthOrganisation. World Tuberculosis Day 2019. Bull World Health Organ. 2019;

72. World Health Organization (WHO). The END TB strategy. World Heal Assem. 2015;

73. WHO. Tuberculosis Fact sheet. Bull World Health Organ. 2018;

74. WHO. Moscow Declaration to End TB. Moscow: Ministry of Health of the Russian Federation and World Health Organization. 2017;

75. Find Diagnostics. STRATEGY FOR TUBERCULOSIS (and lower respiratory tract Infections) 2015 - 2020. 2014;2015–20. Available from: https://www.finddx.org/wp-content/uploads/2016/02/TB-Strategy-WEB.pdf

76. Fitz-gerald M. Sputum Microscopy The handbook. 2013.

77. Koneman EW, Winn WC, Allen SD, Janda WM, Procop GW, Schrenckenberger PC, et al. Koneman's color atlas and textbook of diagnostic microbiology. J Clin Microbiol. 2009;

78. World Health Organization. Fluorescent light-emitting diode (LED) microscopy for diagnosis of tuberculosis Policy statement. WHO press. 2011.

79. Frieden T. Toman's tuberculosis: case detection, treatment and monitoring. Questions and answers. Toman's tuberculosis: case detection, treatment and monitoring. Questions and answers. 2004.

80. Lumb R, Van Deun A, Bastian I, Fitz-Gerald M. Laboratory diagnosis of tuberculosis by

sputum microscopy. World Health Organization. 2013.

81. Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, Cunningham J, et al. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. Lancet Infectious Diseases. 2006.

82. Acumedia. Lowenstein - jensen medium (7245). Neogen Corp. 2017;(7245):4–5.

83. Siddiqi SH. MGIT For BACTEC ^{TM} MGIT 960 ^{TM} TB System. FIND MGIT Mannual. 2006;

84. Tortoli E, Cichero P, Piersimoni C, Simonetti MT, Gesu G, Nista D. Use of BACTEC MGIT 960 for recovery of mycobacteria from clinical specimens: Multicenter study. J Clin Microbiol. 1999;

85. Hanna BA, Ebrahimzadeh A, Elliott LB, Morgan MA, Novak SM, Rusch-Gerdes S, et al. Multicenter evaluation of the BACTEC MGIT 960 system for recovery of mycobacteria. J Clin Microbiol. 1999;

86. van Zyl-Smit RN, Binder A, Meldau R, Mishra H, Semple PL, Theron G, et al. Comparison of quantitative techniques including Xpert MTB/RIF to evaluate mycobacterial burden. PLoS One. 2011;

87. Cruciani M, Scarparo C, Malena M, Bosco O, Serpelloni G, Mengoli C. Meta-Analysis of BACTEC MGIT 960 and BACTEC 460 TB, with or without Solid Media, for Detection of Mycobacteria. J Clin Microbiol. 2004;

88. Nakiyingi L, Kateete DP, Ocama P, Worodria W, Sempa JB, Asiimwe BB, et al. Evaluation of in-house PCR for diagnosis of smear-negative pulmonary tuberculosis in Kampala, Uganda. BMC Res Notes. 2012;

89. Chakravorty S, Simmons AM, Rowneki M, Parmar H, Cao Y, Ryan J, et al. The New Xpert MTB/RIF Ultra: Improving Detection of Mycobacterium tuberculosis and Resistance to Rifampin in an Assay Suitable for Point-of-Care Testing.

90. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, et al. Rapid Molecular Detection of Tuberculosis and Rifampin Resistance. N Engl J Med. 2010;

91. Detjen AK, DiNardo AR, Leyden J, Steingart KR, Menzies D, Schiller I, et al. Xpert MTB/RIF assay for the diagnosis of pulmonary tuberculosis in children: A systematic

review and meta-analysis. Lancet Respir Med. 2015;

92. Cazabon D, Suresh A, Oghor C, Qin ZZ, Kik S V., Denkinger CM, et al. Implementation of Xpert MTB/RIF in 22 high tuberculosis burden countries: are we making progress? The European respiratory journal. 2017.

93. Corrections: Xpert MTB/RIF Ultra for detection of Mycobacterium tuberculosis and rifampicin resistance: a prospective multicentre diagnostic accuracy study (The Lancet Infectious Diseases (2018) 18(1) (76–84)(S1473309917306916)(10.1016/S1473-3099(17)30691-6)). The Lancet Infectious Diseases. 2018.

94. Alland D, Rowneki M, Smith L, Ryan J, Chancellor M, Simmons AM, et al. Xpert MTB/RIF Ultra: A New Near-Patient TB Test With Sensitivity Equal to Culture. In: Conference on Retroviruses and Opportunistic Infections [Internet]. 2015. p. 1. Available from: http://www.croiconference.org/sessions/xpert-mtbrif-ultra-new-near-patient-tb-test-sensitivity-equal-culture

95. Dorman SE, Schumacher SG, Alland D, Nabeta P, Armstrong DT, King B, et al. Xpert MTB/RIF Ultra for detection of Mycobacterium tuberculosis and rifampicin resistance: a prospective multicentre diagnostic accuracy study. Lancet Infect Dis. 2018;

96. WHO. The use of loop-mediated isothermal amplification (TB-LAMP) for the diagnosis of pulmonary tuberculosis; Policy guidance. 2016.

97. Minion J, Leung E, Talbot E, Dheda K, Pai M, Menzies D. Diagnosing tuberculosis with urine lipoarabinomannan: Systematic review and meta-analysis. Vol. 38, European Respiratory Journal. 2011. p. 1398–405.

98. Aung ZZ, Saw YM, Saw TN, Oo N, Aye HNN, Aung S, Oo HN, Cho SM, Khaing M, Kariya T, Yamamoto E HN. Survival rate and mortality risk factors among TB-HIV co-infected patients at an HIV-specialist hospital in Myanmar: A 12-year retrospective follow-up study. Int J Infect Dis. 2019;(80):10–5.

99. WHO. The use of lateral flow urine lipoarabinomannan assay (LF-LAM) for the diagnosis and screening of active tuberculosis in people living with HIV: Policy guidance [Internet]. 2015. Available from: WHO/HTM/TB/2015.25

100. Nicol MP, Allen V, Workman L, Isaacs W, Munro J, Pienaar S, et al. Urine

lipoarabinomannan testing for diagnosis of pulmonary tuberculosis in children: A prospective study. Lancet Glob Heal. 2014;

101.   WHO. Commercial serodiagnostic tests for diagnosis of tuberculosis: policy statement. Geneva WHO. 2011;

102.   McNerney R, Wondafrash BA, Amena K, Tesfaye A, McCash EM, Murray NJ. Field test of a novel detection device for Mycobacterium tuberculosis antigen in cough. BMC Infect Dis. 2010;

103.   Camilleri D. A breath screen for active tuberculosis at the point-of-care.

104.   Kim JY, Kang YA, Park JH, Cha HH, Jeon NY, Lee SW, Lee SO, Choi SH, Kim YS, Woo JH KS. An IFN-γ and TNF-α dual release fluorospot assay for diagnosing active tuberculosis. Clin Microbiol Infect. 2019;(12).

105.   CDC. Fact Sheets | Testing & Diagnosis | Testing for Tuberculosis (TB) | TB | CDC. Centers for Disease Control and Prevention. 2016.

106.   Datta S, Shah L, Gilman RH, Evans CA. Comparison of sputum collection methods for tuberculosis diagnosis: a systematic review and pairwise and network meta-analysis. Lancet Glob Heal. 2017;

107.   Mase SR, Ramsay A, Ng V, Henry M, Hopewell PC, Cunningham J, et al. Yield of serial sputum specimen examinations in the diagnosis of pulmonary tuberculosis: A systematic review. International Journal of Tuberculosis and Lung Disease. 2007.

108.   Castro AT e., Mendes M, Freitas S, Roxo PC. Diagnostic yield of sputum microbiological analysis in the diagnosis of pulmonary tuberculosis in a period of 10 years. Rev Port Pneumol . 2015;

109.   WHO. Same-day diagnosis of tuberculosis by microscopy: Policy Statement. World Heal Organ Doc. 2011;

110.   Walters E, van der Zalm MM, Demers A-M, Whitelaw A, Palmer M, Bosch C, et al. Specimen Pooling as a Diagnostic Strategy for Microbiologic Confirmation in Children with Intrathoracic Tuberculosis. Pediatr Infect Dis J. 2019;

111.   Parashar D, Kabra SK, Lodha R, Singh V, Mukherjee A, Arya T, et al. Does neutralization of gastric aspirates from children with suspected intrathoracic tuberculosis affect

mycobacterial yields on MGIT culture? J Clin Microbiol. 2013;

112. Ioos V, Cordel H, Bonnet M. Alternative sputum collection methods for diagnosis of childhood intrathoracic tuberculosis: A systematic literature review. Arch Dis Child. 2019;

113. Ruiz Jiménez M, Guillén Martín S, Prieto Tato LM, Cacho Calvo JB, Álvarez García A, Soto Sánchez B, et al. " Induced sputum versus gastric lavage for the diagnosis of pulmonary tuberculosis in children." BMC Infect Dis. 2013;

114. Lobato MN, Loeffler AM, Furst K, Cole B, Hopewell PC. Detection of Mycobacterium tuberculosis in gastric aspirates collected from children: hospitalization is not necessary. Pediatrics. 1998;

115. Lopez-Varela E, Garcia-Basteiro AL, Augusto OJ, Fraile O, Bulo H, Ira T, et al. High rates of non-tuberculous mycobacteria isolation in mozambican children with presumptive tuberculosis. PLoS One. 2017;

116. Paggiaro PL, Chanez P, Holz O, Ind PW, Djukanović R, Maestrelli P, et al. Sputum induction. In: European Respiratory Journal, Supplement. 2002.

117. Zar HJ, 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;

118. Li LM, Bai LQ, Yang HL, Xiao CF, Tang RY, Chen YF, et al. Sputum induction to improve the diagnostic yield in patients with suspected pulmonary tuberculosis. Int J Tuberc Lung Dis. 1999;

119. Programme GT. Automated real-time nucleic acid amplification technology for rapid and simultaneous detection of tuberculosis and rifampicin resistance: Xpert MTB/RIF assay for the diagnosis of pulmonary and extrapulmonary TB in adults and children. 2014; Available from: www.who.int/tb

120. Marcy O, Ung V, Goyet S, Borand L, Msellati P, Tejiokem M, et al. Performance of Xpert MTB/RIF and Alternative Specimen Collection Methods for the Diagnosis of Tuberculosis in HIV-Infected Children. Clin Infect Dis. 2016;

121. Owens S, Abdel-Rahman IE, Balyejusa S, Musoke P, Cooke RPD, Parry CM, et al. Nasopharyngeal aspiration for diagnosis of pulmonary tuberculosis. Arch Dis Child. 2007;

122. MacLean E, Sulis G, Denkinger CM, Johnston JC, Pai M AKF. Diagnostic accuracy of stool Xpert MTB/RIF for detection of pulmonary tuberculosis in children: a systematic review and metaanalysis. J Clin Microbiol. 2019;

123. Benjelloun A, Darouassi Y, Zakaria Y, Bouchentouf R, Errami N. Lymph nodes tuberculosis: A retrospective study on clinical and therapeutic features. Pan Afr Med J. 2015;

124. Perez-Velez CM, Marais BJ. Tuberculosis in Children. N Engl J Med. 2012;

125. Denkinger CM, Schumacher SG, Boehme CC, Dendukuri N, Pai M SK. Xpert MTB/RIF assay for the diagnosis of extrapulmonary tuberculosis: a systematic review and meta-analysis. Eur Respir J. 2014;44(2):435–46.

126. Chen Y, Wu P, Fu L, Liu YH, Zhang Y ZY. Multicentre evaluation of Xpert MTB/RIF assay in detecting urinary tract tuberculosis with urine samples. Sci Rep. 2019;30(9):1.

127. Luabeya AK, Wood RC, Shenje J, Filander E, Ontong C, Mabwe S, et al. Noninvasive detection of tuberculosis by oral swab analysis. J Clin Microbiol. 2019;57(3):12–5.

128. Cepheid. Operator Manual test Xpert MTB / RIF. 2009.

129. Reeve BWP, McFall SM, Song R, Warren R, Steingart KR, Theron G. Commercial products to preserve specimens for tuberculosis diagnosis: a systematic review. Int J Tuberc Lung Dis. 2018;

130. Selvakumar N, Vanajakumar, Narayana ASL, Suryanarayanan D, Umapathy KC, Paramasivan CN, et al. Use of cetylpyridinium chloride for storage of sputum specimens and isolation of M. tuberculosis. Indian J Tuberc. 1993;

131. Phillips BJ, Kaplan W. Effect of cetylpyridinium chloride on pathogenic fungi and Nocardia asteroides in sputum. J Clin Microbiol. 1976;

132. Selvakumar N, Gomathi Sekar M, Ilampuranan KJ, Ponnuraja C, Narayanan PR. Increased detection by restaining of acid-fast bacilli in sputum samples transported in cetylpyridinium chloride solution. Int J Tuberc Lung Dis. 2005;

133. Shinu P, Singh V, Nair A, Mehrishi P, Mehta S, Joshi E. Application of cetylpyridinium chloride and sodium chloride decontamination method for recovery of Mycobacterium tuberculosis from clinically suspected cases of pulmonary tuberculosis. Diagn Microbiol

Infect Dis. 2013;

134. Pal N, Sharma B, Malhotra B, Rishi S. Transport and storage of sputum specimen by using cetylpyridinium chloride for isolation of mycobacteria. Indian J Pathol Microbiol. 2009;

135. Hiza H, Doulla B, Sasamalo M, Hella J, Kamwela L, Mhimbira F, et al. Preservation of sputum samples with cetylpyridinium chloride (CPC) for tuberculosis cultures and Xpert MTB/RIF in a low-income country. BMC Infect Dis. 2017;

136. Affolabi D, Sanoussi N, Sossou A, Nys T, Bodi O, Esse M, et al. Performance of OMNIgene•SPUTUM (DNA Genotek) and cetylpyridinium chloride for sputum storage prior to mycobacterial culture. J Med Microbiol. 2018;

137. Sankar MM, Kumar P, Munawwar A, Singh J, Parashar D, Singh S. Recovery of Mycobacterium tuberculosis from sputum treated with cetyl pyridinium chloride. Journal of Clinical Microbiology. 2009.

138. Williams DL, Gillis TP, Dupree WG, Hansen GWL. Ethanol fixation of sputum sediments for DNA-based detection of Mycobacterium Updated information and services can be found at : These include : Ethanol Fixation of Sputum Sediments for DNA-Based Detection of Mycobacterium tuberculosis. 1995;33(6).

139. Chedore P, Th'ng C, Nolan DH, Churchwell GM, Sieffert DE, Hale YM, et al. Method for inactivating and fixing unstained smear preparations of mycobacterium tuberculosis for improved laboratory safety. J Clin Microbiol. 2002;

140. Maharjan B, Shrestha B, Weirich A, Stewart A, Kelly-Cirino CD. A novel sputum transport solution eliminates cold chain and supports routine tuberculosis testing in Nepal. J Epidemiol Glob Health. 2016;

141. Nambiar R, Shaw K, Curry PS. Evaluation of OMNIgene®•SPUTUM for tuberculosis sample processing and liquid culture at a hospital laboratory in India. J Microbiol Methods. 2017;

142. Kelly-Cirino CD, Musisi E, Byanyima P, Kaswabuli S, Andama A, Sessolo A, et al. Investigation of OMNIgene·SPUTUM performance in delayed tuberculosis testing by smear, culture, and Xpert MTB/RIF assays in Uganda. J Epidemiol Glob Health.

2017;7(2):103–9.

143. Tagliani E, Alagna R, Tafaj S, Hafizi H, Cirillo DM. Evaluation of Mycobacterium tuberculosis viability in OMNIgene-SPUTUM reagent upon multi-day transport at ambient temperature. BMC Infect Dis. 2017;

144. Azam K, Cadir N, Madeira C, Gillespie SH, Sabiiti W. OMNIgene.SPUTUM suppresses contaminants while maintaining *Mycobacterium tuberculosis* viability and obviates cold-chain transport. ERJ Open Res. 2018;4(1):00074-2017.

145. Zallet J, Olaru ID, Witt A, Vock P, Kalsdorf B, Andres S, et al. Evaluation of OMNIgene W SPUTUM reagent for mycobacterial culture. 2018;22(January):945–9.

146. Maharjan B, Kelly-Cirino CD, Weirich A, Curry PS, Hoffman H, Avsar K, et al. Evaluation of OMNIgene W SPUTUM-stabilised sputum for long- term transport and Xpert W MTB/RIF testing in Nepal. INT J TUBERC LUNG DIS. 2016;

147. Sanoussi CN, de Jong BC, Affolabi D, Meehan CJ, Odoun M RL. Storage of sputum in cetylpyridinium chloride, OMNIgeneSPUTUM and ethanol is compatible with molecular tuberculosis diagnostic testing. J Clin Microbiol. 2019;15(19).

148. Del Castillo-Barrientos H, Centeno-Luque G, Untiveros-Tello A, Simms B, Lecca L, Nelson AK, et al. Clinical presentation of children with pulmonary tuberculosis: 25 years of experience in Lima, Peru. Int J Tuberc Lung Dis. 2014;

149. Marais BJ, Gie RP, Hesseling AC, Schaaf HS, Lombard C, Enarson DA, et al. A Refined Symptom-Based Approach to Diagnose Pulmonary Tuberculosis in Children. Pediatrics. 2006;

150. Hesseling AC, Schaaf HS, Gie RP, Starke JR, Beyers N. A critical review of diagnostic approaches used in the diagnosis of childhood tuberculosis. International Journal of Tuberculosis and Lung Disease. 2002.

151. Edwards DJ, Kitetele F, Van Rie A. Agreement between clinical scoring systems used for the diagnosis of pediatric tuberculosis in the HIV era. Int J Tuberc Lung Dis. 2007;

152. Oliwa JN, Karumbi JM, Marais BJ, Madhi SA, Graham SM. Tuberculosis as a cause or comorbidity of childhood pneumonia in tuberculosis-endemic areas: A systematic review. The Lancet Respiratory Medicine. 2015.

153. Marais BJ, Graham SM. Symptom-based screening of child TB contacts: Defining "symptomatic." International Journal of Tuberculosis and Lung Disease. 2017.

154. Fox GJ, Barry SE, Britton WJ, Marks GB. Contact investigation for tuberculosis: A systematic review and meta-analysis. European Respiratory Journal. 2013.

155. Szkwarko D, Hirsch-Moverman Y, Du Plessis L, Du Preez K, Carr C, Mandalakas AM. Child contact management in high tuberculosis burden countries: A mixed-methods systematic review. PLoS One. 2017;

156. Graham SM, Cuevas LE, Jean-Philippe P, Browning R, Casenghi M, Detjen AK, et al. Clinical Case Definitions for Classification of Intrathoracic Tuberculosis in Children: An Update. Clin Infect Dis. 2015;

157. Uganda Bureau of Statistics. National Population and Housing Census 2014. Uganda Bureau of Statistics. 2016.

158. World Health Organisation. WHO/TheUnion/Unicef/CDC/USAID/TAG. Roadmap for childhood tuberculosis: towards zero deaths. [Internet]. Accessed 3 September 2019. 2013. Available from: http://apps.who.int/iris/bitstream/10665/89506/1/9789241506137_eng.pdf.

159. Graham SM, Ahmed T, Amanullah F, Browning R, Cardenas V, Casenghi M, et al. Evaluation of tuberculosis diagnostics in children: 1. Proposed clinical case definitions for classification of intrathoracic tuberculosis disease. Consensus from an expert panel. J Infect Dis. 2012;

160. Nicol MP, Spiers K, Workman L, Isaacs W, Munro J, Black F, et al. Xpert MTB/RIF testing of stool samples for the diagnosis of pulmonary tuberculosis in children. Clin Infect Dis. 2013;

161. Lacourse SM, Pavlinac PB, Cranmer LM, Njuguna IN, Mugo C, Gatimu J, et al. Stool Xpert MTB/RIF and urine lipoarabinomannan for the diagnosis of tuberculosis in hospitalized HIV-infected children. AIDS. 2018;

162. DiNardo AR, Hahn A, Leyden J, Stager C, Baron EJ, Graviss EA, et al. Use of string test and stool specimens to diagnose pulmonary tuberculosis. Int J Infect Dis. 2015;

163. Jenkins HE, Yuen CM, Rodriguez CA, Nathavitharana RR, McLaughlin MM, Donald P,

et al. Mortality in children diagnosed with tuberculosis: a systematic review and meta-analysis. Lancet Infect Dis. 2017;17(3).

164. Hailu D, Abegaz WE, Belay M. Childhood tuberculosis and its treatment outcomes in Addis Ababa: A 5-years retrospective study. BMC Pediatr. 2014;

165. Lolekha R, Anuwatnonthakate A, Nateniyom S, Sumnapun S, Yamada N, Wattanaamornkiat W, et al. Childhood TB epidemiology and treatment outcomes in Thailand: A TB active surveillance network, 2004 to 2006. BMC Infect Dis. 2008;

166. Graham SM, Sismanidis C, Menzies HJ, Marais BJ, Detjen AK, Black RE. Importance of tuberculosis control to address child survival. The Lancet. 2014.

167. Graham SM. Research into tuberculosis diagnosis in children. The Lancet Infectious Diseases. 2010.

168. Setel PW, Whiting DR, Hemed Y, Chandramohan D, Wolfson LJ, Alberti KGMM, et al. Validity of verbal autopsy procedures for determining cause of death in Tanzania. Trop Med Int Heal. 2006;

169. Teklu AM, Nega A, Mamuye AT, Sitotaw Y, Kassa D, Mesfin G, et al. Factors Associated with Mortality of TB/HIV Co-infected Patients in Ethiopia. Ethiop J Health Sci. 2017;

170. Union. Priorities for tuberculosis bacteriology services in low-income countries. 2007;

171. Landis JR, Koch GG. The Measurement of Observer Agreement for Categorical Data. Biometrics. 1977;

172. Nicol MP, Workman L, Isaacs W, Munro J, Black F, Eley B, et al. Accuracy of the Xpert MTB/RIF test for the diagnosis of pulmonary tuberculosis in children admitted to hospital in Cape Town, South Africa: A descriptive study. Lancet Infect Dis. 2011;

173. Mesman AW, Soto M, Coit J, Calderon R, Aliaga J, Pollock NR, et al. Detection of Mycobacterium tuberculosis in pediatric stool samples using TruTip technology. BMC Infect Dis. 2019;

174. Walters E, van der Zalm MM, Palmer M, Bosch C, Demers A-M, Draper H, et al. Xpert MTB/RIF on Stool Is Useful for the Rapid Diagnosis of Tuberculosis in Young Children With Severe Pulmonary Disease. Pediatr Infect Dis J. 2017;

175. Chipinduro M, Mateveke K, Makamure B, Ferrand RA, Gomo E. Stool Xpert ® MTB/RIF test for the diagnosis of childhood pulmonary tuberculosis at primary clinics in Zimbabwe . Int J Tuberc Lung Dis. 2016;

176. Hasan Z, Shakoor S, Arif F, Mehnaz A, Akber A, Haider M, et al. Evaluation of Xpert MTB/RIF testing for rapid diagnosis of childhood pulmonary tuberculosis in children by Xpert MTB/RIF testing of stool samples in a low resource setting. BMC Res Notes. 2017;

177. Walters E, Scott L, Nabeta P, Demers AM, Reubenson G, Bosch C, et al. Molecular detection of mycobacterium tuberculosis from stools in young children by use of a novel centrifugation-free processing method. J Clin Microbiol. 2018;

178. Iskandar A, Nursiloningrum E, Arthamin MZ, Olivianto E, Chandrakusuma MS. The diagnostic value of urine lipoarabinomannan (LAM) antigen in childhood tuberculosis. J Clin Diagnostic Res. 2017;

179. Kroidl I, Clowes P, Mwakyelu J, Maboko L, Kiangi A, Rachow A, et al. Reasons for false-positive lipoarabinomannan ELISA results in a Tanzanian population. Scand J Infect Dis. 2014;

180. Dheda K, Davids V, Lenders L, Roberts T, Meldau R, Ling D, et al. Clinical utility of a commercial LAM-ELISA assay for TB diagnosis in HIV-infected patients using urine and sputum samples. PLoS One. 2010;

181. Lawn SD. Point-of-care detection of lipoarabinomannan (LAM) in urine for diagnosis of HIV-associated tuberculosis: A state of the art review. BMC Infectious Diseases. 2012.

182. Gounder CR, Kufa T, Wada NI, Mngomezulu V, Charalambous S, Hanifa Y, et al. Diagnostic accuracy of a urine lipoarabinomannan enzyme-linked immunosorbent assay for screening ambulatory HIV-infected persons for tuberculosis. J Acquir Immune Defic Syndr. 2011;

183. Broger T, Sossen B, du Toit E, Kerkhoff AD, Schutz C, Ivanova Reipold E, et al. Novel lipoarabinomannan point-of-care tuberculosis test for people with HIV: a diagnostic accuracy study. Lancet Infect Dis. 2019;

184. Siddiqi SH. MGIT TM Procedure Manual for BACTEC TM MGIT 960 TM TB System. System. 2006;(July).

185. Maharjan B, Kelly-Cirino CD, Weirich A, Curry PS, Hoffman H, Avsar K, et al. Evaluation of OMNIgene®•SPUTUM-stabilised sputum for long-term transport and Xpert® MTB/RIF testing in Nepal. Int J Tuberc Lung Dis. 2016;

186. Asandem DA, Asante-Poku A, Asare P, Aboagye SY, Stephen O-W, Danso E, et al. OMNIgene SPUTUM: A good transport and decontaminating reagent for tuberculosis testing. Int J Mycobacteriology. 2018;

187. Walters E, Gie RP, Hesseling AC, Friedrich SO, Diacon AH, Gie RP. Rapid diagnosis of pediatric intrathoracic tuberculosis from stool samples using the xpert MTB/RIF assay: A pilot study. Pediatric Infectious Disease Journal. 2012.

188. Walters E, Demers A-M, van der Zalm MM, Whitelaw A, Palmer M, Bosch C, et al. Stool culture for the diagnosis of pulmonary tuberculosis in children. J Clin Microbiol. 2017;

189. Banada PP, Naidoo U, Deshpande S, Karim F, Flynn JL, O'Malley M, et al. A novel sample processing method for rapid detection of tuberculosis in the stool of pediatric patients using the Xpert MTB/RIF assay. PLoS One. 2016;

190. Lorent N, Choun K, Malhotra S, Koeut P, Thai S, Khun KE et al. Challenges from Tuberculosis Diagnosis to Care in Community-Based Active Case Finding among the Urban Poor in Cambodia: A Mixed-Methods Study. PLoS One. 2015;10(7).

191. Reid MJ, Saito S, Fayorsey R et al. Assessing capacity for diagnosing tuberculosis in children in sub-Saharan African HIV care settings. Int J Tuberc Lung Dis. 2012;16:924–7.

192. Haile Welday S, Nyerere Kimang A, Muthoni Kabera B, Wahogo Mburu J, Mwachari C, Mungai E, et al. Stool as Appropriate Sample for the Diagnosis of Mycobacterium tuberculosis by Gene Xpert Test. Open J Respir Dis [Internet]. 4:83–9. Available from: http://www.scirp.org/journal/ojrd

193. Andriyoko B, Janiar H, Kusumadewi R, Klinkenberg E, de Haas P, Tiemersma E. Simple stool processing method for the diagnosis of pulmonary tuberculosis using GeneXpert MTB/RIF. The European respiratory journal. 2019.

194. Sabi I, Rachow A, Mapamba D, Clowes P, Ntinginya NE, Sasamalo M, et al. Xpert MTB/RIF Ultra assay for the diagnosis of pulmonary tuberculosis in children: a multicentre comparative accuracy study. J Infect. 2018;77(4).

195. Nicol MP, Workman L, Prins M, Bateman L, Ghebrekristos Y, Mbhele S, Denkinger CM ZH. Accuracy of Xpert Mtb/Rif Ultra for the Diagnosis of Pulmonary Tuberculosis in Children. Pediatr Infect Dis J. 2018;37(10).

196. Nel JS, Lippincott CK, Berhanu R, Spencer DC, Sanne IM, Ive P. Does Disseminated Nontuberculous Mycobacterial Disease Cause False-Positive Determine TB-LAM Lateral Flow Assay Results? A Retrospective Review. Clin Infect Dis. 2017;

197. Joloba M, Mwangi C, Alexander H, Nadunga D, Bwanga F, Modi N, et al. Strengthening the Tuberculosis Specimen Referral Network in Uganda: The Role of Public-Private Partnerships. J Infect Dis. 2016;

198. Latorre I, Díaz J, Mialdea I, Serra-Vidal M, Altet N, Prat C, et al. IP-10 is an accurate biomarker for the diagnosis of tuberculosis in children. J Infect. 2014 Dec;69(6):590–9.

199. Choudhary RK, Wall KM, Njuguna I, Pavlinac PB, Lacourse SM, Otieno V, et al. Monocyte-to-lymphocyte ratio is associated with tuberculosis disease and declines with Anti-TB Treatment in HIV-infected children. J Acquir Immune Defic Syndr. 2019;

200. Guadagnino G, Serra N, Colomba C, Giammanco A, Mililli D, Scarlata F, et al. Monocyte to lymphocyte blood ratio in tuberculosis and HIV patients: Comparative analysis, preliminary data. Pharmacologyonline. 2017;

201. Anderson ST, Kaforou M, Brent AJ, Wright VJ, Banwell CM, Chagaluka G, et al. Diagnosis of childhood tuberculosis and host RNA expression in Africa. N Engl J Med. 2014;

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PhD Training

Certified short trainings

Mar 2019:   Research Methods Course, Mwanza, Tanzania

Feb 2019:   Grant writing, Mbarara University of Science and Tech (MUST), Uganda

Dec 2018:   Good Clinical and Lab Practices, Epicentre, Uganda

Dec 2018:   TB speed International study implementation, Kampala, Uganda

Aug 2018:   Responsible Conduct of Research, MUST, Uganda

June 2017:   TB Research Methods, McGill University, Canada

June 2017:   Advanced TB methods course. McGill University, Canada

April 2017:   Field Management and Leadership Training (FMT), Epicentre, Uganda

Oral presentations

Nov 2018:   Accuracy of two non-sputum based tuberculosis diagnostic tests in children at increased risk of severe TB, MUST-ARDC, Uganda

Oct 2018:   Evaluation of the Omnigene reagent for long term preservation of sputum for MGIT culture in Uganda, Union conference, Hague, Netherlands

Aug 2018:   xpert diagnosis of childhood TB from sputum and stool in high TB-HIV prevalent settings, Uganda Pediatric Association, Kampala, Uganda

Nov 2017:   Evaluation of OMNIgene® for long term preservation of samples for Xpert testing, UMLTA Annual Scientific Conference, Arua, Uganda

Nov 2017:   Evaluation of OMNIgene® for long term preservation of samples for Xpert testing, MUST-Annual Research Dissemination conference, Uganda

Jan 2017:   New Developments in Our Basic Understanding of Tuberculosis, Canada

Poster presentations

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Lecturing

2016-todate: Laboratory Quality Management System and Lab Ethics. Postgraduate students of MSc Microbiology, Mbarara University

2016-date   Honorary Lecturer, Undergraduate Medical students, Faculty of Medicine, MUST

Supervisory role

2016-17:   Charles Suuna; MSc, Microbiology — Use of OMNIgene® to increase time delay between sample collection and testing with Xpert

2018-19:   Allan T Muhumuza; MSc, Pharmacoloy — In vitro evaluation of Erythrina abyssinica and Lantana camara formulation against Mycobacterium tuberculosis strains

ABOUT THE AUTHOR

Patrick Orikiriza was born in Mbarara District in Southwestern Ugandan. He went to Bishop Stuart Primary School, St. Joseph's Vocational School for post primary studies. He won a government scholarship to pursue a diploma in Medical Laboratory Technology, at Makerere University, where he majored in Clinical Microbiology. During diploma studies he got so passionate about research and despite minimum resources, investigated prevalence of TB among HIV patients at Mulago National Referral Hospital. This was among the first studies to highlight TB-HIV coinfection and it opened widespread interest in this population. Indeed, this research work attracted several interests in the author by prominent research organizations including tenure at Uganda Virus Research Institute where he participated in the national HIV sero-survey. He decided to join Joint Clinical Research Centre under the Case Western Reserve University Collaboration at the main station in Kampala. He was so much involved in TB research work and was later identified due to his technical competence to pursue an advanced course in molecular technology at the University of Arkansas for Medical Sciences in the United States. He went back to school to pursue a Bachelor of Science degree in order to learn and update his skills.

After sometime, he chose to return to his home district of Mbarara in order to venture deeper into research writing, publication as well as take up some management responsibilities with Epicentre. At Epicentre, he enrolled for an MSc in Microbiology at Mbarara University of Science and Technology, where he excelled and was retained as an honorary lecturer in the faculty. He has also played a major role in several TB related studies including principal investigator on some diagnostic studies. The author enrolled for PhD in 2016 and was honored to be directed by Dr. Maryline Bonnet one of the best ever mentors and supervisors. The author believes that this PhD has imparted in him more research skills to write, train and innovate. With this accolade, the author believes he can will contribute better in improving the living conditions of the poor. He hopes to devote more time in research and training but also in strengthening laboratory systems in resource limited settings.