

**Accuracy of smear microscopy and incremental yield of Xpert MTB/RIF in  
detection of pulmonary tuberculosis in laboratories in Rwanda**

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**A dissertation submitted in part fulfillment for the requirements for the  
degree of Master of Science in Medical Microbiology at the University of**

**Nairobi,**

**October 2014**

**DECLARATION**

I certify that this dissertation is my original work done with guidance of my supervisors and to the best of my knowledge has not been presented for a degree at any other university.

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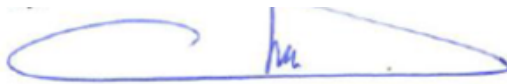
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## **DEDICATION**

To my beloved family

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## ACRONYMS

|                                |  |
|--------------------------------|--|
| <b>AFB</b>                     | Acid Fast Bacilli  |
| <b>BCG</b>                     | Bacille Calmette-Guerin  |
| <b>BD</b>                      | Becton Dickinson Company   |
| <b>CDC</b>                     | Center for Diseases Control and prevention                                   |
| <b>CRI</b>                     | Colorimetric Redox Indicator   |
| <b>DNA</b>                     | Deoxyribonucleic Acid  |
| <b>DOTS</b>                    | Directly Observed Therapy Short-course                                       |
| <b>DST</b>                     | Drug Susceptibility Testing  |
| <b>E</b>                       | Ethambutol   |
| <b>ELISPOT</b>                 | Enzyme-Linked Immunospot assay   |
| <b>FIND</b>                    | Foundation for Innovative New Diagnostics                                    |
| <b>FM</b>                      | Fluorescence Microscopy  |
| <b>HIV</b>                     | Human Immunodeficiency Virus   |
| <b>HPLC</b>                    | High performance Liquid Chromatography                                       |
| <b>IFN-<math>\gamma</math></b> | Interferon-gamma   |
| <b>IGRA</b>                    | Interferon-gamma Release Assay   |
| <b>INH</b>                     | Isoniazid  |
| <b>IUATLD</b>                  | International Union Against Tuberculosis and Lung Disease                    |
| <b>KNH/UoN-ERC</b>             | Kenyatta National Hospital/University of Nairobi- Ethical Research Committee |
| <b>LED-FM</b>                  | Light Emitting Diode Fluorescence Microscopy                                 |
| <b>LJ</b>                      | Löwenstein-Jensen  |
| <b>LPA</b>                     | Line Probe Assays  |
| <b>MDR-TB</b>                  | Multi-Drug Resistant Tuberculosis  |
| <b>MGIT</b>                    | Mycobacterium Growth Indicator Tube  |
| <b>MODS</b>                    | Microscopic Observation of Drug Susceptibility                               |
| <b>MTB</b>                     | <i>Mycobacterium tuberculosis</i> complex                                    |
| <b>NAAT</b>                    | Nucleic Acid Amplification Test  |
| <b>NALC-NaOH</b>               | Sodium Hydroxide N- acetyl- Cystein solution                                 |
| <b>NTM</b>                     | Non Tuberculosis Mycobacterium   |

|               |  |
|---------------|--|
| <b>NTP</b>    | National Tuberculosis Control Program    |
| <b>PCR</b>    | Polymerase Chain Reaction                |
| <b>pH</b>     | Hydrogen ion concentration               |
| <b>PPD-S</b>  | Standardized Purified Protein Derivative |
| <b>RIF</b>    | Rifampicin                               |
| <b>S</b>      | Streptomycin                             |
| <b>TB</b>     | Tuberculosis                             |
| <b>WHO</b>    | World Health Organization                |
| <b>XDR-TB</b> | Extensively Drug-Resistant Tuberculosis  |
| <b>Z</b>      | Pyrazinamide                             |
| <b>ZN</b>     | Ziehl Neelsen staining                   |

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## **ABSTRACT**

**Background:** The National tuberculosis control Program (NTP) of Rwanda is currently phasing in the use of light emitting diode-fluorescent microscopy (LED-FM) as an alternative to the Ziehl-Neelsen technique (ZN). This, alongside the Xpert MTB/RIF technique is expected to improve diagnosis of tuberculosis (TB) and detection of Rifampicin resistance in patients at the various health facilities. There is limited information in Rwanda on the incremental yield of these new techniques in comparison to the ZN technique which has been in use for many years.

**Objective:** To determine the accuracy of routine sputum smear microscopy and assess the incremental yield of Xpert MTB/RIF test in detection of pulmonary tuberculosis in peripheral and intermediate health facility laboratories in Rwanda.

**Methods:** The study was conducted at four intermediate and four peripheral health facility laboratories across Rwanda. This study enrolled 648 participants with suspected pulmonary tuberculosis who visited any of the eight health facilities from April 1<sup>st</sup> to June 21<sup>st</sup>, 2014. Each participant gave one sputum sample which was processed as per the individual laboratory procedures and the results obtained from the direct sputum smear recorded. The remaining sample was processed using N-acetyl-cystein-Sodium hydroxide and inoculated a Lowenstein Jensen slant and mycobacterium growth indicator tube (MGIT) at the National Reference Laboratory (NRL). The pellet was used to prepare two smears, one for ZN and the other for LED-FM staining and microscopy. The original health facility slide was re-examined at NRL and the results recorded. The pellet was also used for testing using Xpert MTB/RIF.

**Results:** The study found the overall sensitivity and specificity of smear microscopy to be 51.5% and 99.8% respectively, and positive and negative predictive values of 98% and 91.4% respectively. The increment of Xpert MTB/RIF test over the smear microscopy was 40.3% and

47.6% respectively in HIV-negative and HIV-positive tuberculosis suspects. The overall prevalence of smear negative pulmonary tuberculosis was 39.2%.

**Conclusion:** This study has provided the baseline data on the performance of currently used routine methods and the incremental yield of Xpert MTB/RIF test compared to MTB culture as the gold standard. It is therefore imperative to implement measures which could increase the sensitivity of LED-FM microscopy before replacing ZN techniques in health facility laboratories. The use of smear microscopy alone misses more than 50% of pulmonary tuberculosis cases, consequently all smear negative cases with prominent signs and symptoms of pulmonary tuberculosis should be tested using Xpert MTB/RIF test in order to increase tuberculosis case detection and reduce the chain of transmission.

## **OPERATIONAL DEFINITIONS**

**Sputum smear microscopy** – involves collecting a sputum specimen, fixing it thinly on a glass slide and then staining it with a dye (carbol fuchsin or auramine-rhodamine staining) that binds specifically to mycobacteria; making them easier to identify under a microscope. Smear microscopy for TB is sometimes called AFB testing, because mycobacteria are ‘acid-fast bacilli’ (AFB). This means that certain dyes adhere to the waxy coat of mycobacteria and remain visible even after rinsing with water and being briefly treated with a solution of acid-alcohol that strips the dye from the rest of the smear [1].

**Accuracy** – is the condition or quality of being true, correct, or exact. It is an extent to which a given measurement agrees with the standard value for that measurement. In this study, results from culture were considered as gold standard and the correctness of sputum smear microscopy results has been assessed [2].

**Intermediate health facility laboratories in Rwanda** – also called district hospitals laboratories, have a center for diagnosis and treatment of tuberculosis. These laboratories use smear microscopy method (ZN or LED-FM) and the staffs are supervised, mentored and trained by the NRL. The district hospital laboratories supervise and train peripheral health facility laboratories. These laboratories transfer sputum specimens suspected to have multidrug resistant TB to the NRL for culture and susceptibility testing.

**Peripheral health facility laboratories in Rwanda** – also called health center laboratories, are the lowest level of public health facility laboratories. More than 70% of them have a center for diagnosis and treatment of TB which use smear microscopy method (ZN or LED-FM). They also

have a referral system for multidrug resistant TB suspected cases to the NRL for culture and susceptibility testing.



## **1.0. INTRODUCTION**

### **1.1. Background**

Sputum smear microscopy is currently recommended for the diagnosis of pulmonary tuberculosis in low and middle income countries, where more than 90% of tuberculosis (TB) cases occur. Microscopy is rapid, relatively simple, inexpensive, and highly specific in areas where there is a high prevalence of tuberculosis [3]. In addition, microscopy identifies the most infectious patients and is widely applicable. However, it has several limitations including the fact that it is examiner-, technique-, and prevalence-dependent and in addition, it lacks sensitivity [3]. In some studies, microscopy has been reported to have greater than 80% sensitivity for identifying cases of pulmonary tuberculosis [4]. However, in other reports the sensitivity of the test has been relatively low and variable (range 20–60%) [4]. The sputum smear microscopy method could detect around 70% of pulmonary tuberculosis cases in optimal conditions [5].

The fluorescence microscopy (FM) of auramine-stained smears has been studied as an alternative to conventional light microscopy with Ziehl Neelsen staining (ZN). The FM shows a similar specificity and on average 10% higher sensitivity than ZN staining [4]. However it is not widely implemented in many TB-endemic settings, one reason being the high costs of the microscope. Studies evaluating the performance of Light Emitting Diode Fluorescence Microscopy (LED-FM) have shown that in addition to the higher sensitivity, it had qualitative, operational and cost advantages over both conventional FM and ZN. On the basis of these findings, the World Health Organization (WHO) recommended in 2011 to replace conventional FM with LED-FM and phase in LED-FM as an alternative to ZN microscopy [6].

On the other hand in 2010, WHO recommended that Xpert MTB RIF test be used at district and sub-district levels as the initial diagnostic test in individuals suspected of having MDR-TB or

HIV-associated TB and be considered as a follow-on test for smear-negative patients in other settings[7] based on the high performance of this test and recently the WHO has issued updated recommendations on the use of Xpert MTB/RIF. This new policy guidance widens the recommended use of Xpert MTB/RIF, including for the diagnosis of paediatric TB and on selected specimens for the diagnosis of extra-pulmonary TB, and includes an additional recommendation on the use of Xpert MTB/RIF as the initial diagnostic test in all individuals presumed to have pulmonary TB [8]. The sensitivity and specificity of Xpert MTB RIF test compared with culture were 88% (95% CI 83 to 92) and 98% (97 to 99), respectively, with a sensitivity in smear-positive cases of 98% (97 to 99) and 68% (60 to 75) in smear-negative cases [9].

The NTP of Rwanda has started the phase-in of LED-FM as an alternative to ZN microscopy in peripheral and intermediate health facility laboratories. Up to date 30% of laboratories have implemented the use of LED-FM and 16 health facilities started using Xpert MTB/RIF test for TB detection in 2012. However currently, there is limited data on the accuracy of ZN and LED-FM sputum smear microscopy and incremental yield of Xpert MTB/RIF in detection of pulmonary tuberculosis at peripheral and intermediate health facility laboratories to support scale up of new molecular technology.

In this study the accuracy of sputum smears microscopy and incremental yield of Xpert MTB RIF test in four intermediate and four peripheral health facilities was assessed.

## **1.2. Rationale**

Tuberculosis is one of the most serious infectious diseases and a considerable public health problem due to its high risk of person-to-person transmission, morbidity, and mortality particularly in the context of HIV co infection. Early diagnosis followed by adequate treatment is

essential to prevent both morbidity and mortality. Microscopic observation of *Mycobacterium tuberculosis* in sputum smears still remains the mainstay of TB diagnosis in developing countries despite its poor sensitivity; case detection through quality assured bacteriology is an essential element of the WHO STOP TB Strategy [10].

In low income countries, sputum smear microscopy is the only cost-effective tool for diagnosing patients with infectious TB and to monitor their response to treatment. It yields timely results but the sensitivity is low compared to nucleic acid amplification test (NAAT) and culture. Despite the low sensitivity of sputum smear microscopy method, it is examiner-, technique-, and prevalence-dependent. Optimally the method could detect 70% of all pulmonary tuberculosis cases, but in clinical practice, the proportion of positive sputum smears is around 30-60% [5].

Timely identification of mycobacterial infection is critical to initiate early, accurate and specific treatment, to improve prognosis and to reduce the risk of dissemination. Therefore, a global strategy for the development and strengthening of laboratory diagnosis is urgently needed to improve the case detection rate, especially in regions with high prevalence of TB and HIV[11].

The case detection rate of tuberculosis in Rwanda is estimated to be 60% (54-67%) [12]. This detection rate is still low compared to the target of 70% case detection rate set by WHO STOP TB strategy in 2005 [10]. There is need to increase this detection rate in order to increase proper management and to reduce transmission of tuberculosis infection in communities. Optimization of smear microscopy as the most important diagnostic tool could significantly increase tuberculosis detection rate in Rwandan health facilities settings. In order to increase the accuracy of sputum smear microscopy, adherence to standard laboratory operating procedures (SOPs), properly trained staff and an efficient quality assurance (QA) system are required, in addition to adequate selection of patients and good-quality of sputum samples [13]. This study has provided

the baseline on the performance of current routine methods and the incremental yield of Xpert MTB RIF test compared to MTB culture as the gold standard. The data obtained could be used to effect policy changes, to review the national tuberculosis diagnosis algorithm and to give guidance on the use of the Xpert MTB RIF test for TB diagnosis at intermediate and peripheral health facilities.

### **1.3. Research question**

What are the accuracy of sputum smear microscopy and the increment yield of Xpert MTB/RIF test in detection of pulmonary TB in peripheral and intermediate health facility laboratories in Rwanda?

### **1.4.Objectives of study**

#### **1.4.1. General objective**

To determine the accuracy of routine sputum smear microscopy in detection of pulmonary TB in peripheral and intermediate health facility laboratories and to assess the incremental yield of Xpert MTB/RIF test.

#### **1.4.2. Specific objectives**

1. To determine the sensitivity, specificity and predictive value of sputum smear microscopy in selected laboratories (Rwamagana, Kabgayi, Kicukiro, Ruhengeri, Bilyogo, Cor-Unum, PCK, RMH) in Rwanda
2. To determine the prevalence of sputum smear negative pulmonary TB in selected health facility laboratories in Rwanda (Rwamagana, Kabgayi, Kicukiro, Ruhengeri, Bilyogo, Cor-Unum, PCK, RMH) in Rwanda

3. To assess the sensitivity and specificity of Xpert MTB/RIF test for pulmonary TB detection in selected laboratories in Rwanda (Rwamagana, Kabgayi, Kicukiro, Ruhengeri, Bilyogo, Cor-Unum, PCK, RMH) in Rwanda)

## **2.0.LITERATURE REVIEW**

### **2.1.Biology of tuberculosis**

#### **2.1.1. Causative organism and transmission**

Tuberculosis is an infection caused by the rod-shaped, non-spore-forming, aerobic bacterium *Mycobacterium tuberculosis* [14]. Mycobacteria typically measure 0.5 µm by 3 µm, are classified as acid-fast bacilli, and have a unique cell wall structure crucial to their survival. *M. tuberculosis* (MTB) belongs to the genus *Mycobacterium* that includes more than 80 other species. Tuberculosis is defined as a disease caused by members of the *M. tuberculosis* complex, which includes the tubercle bacillus (*M. tuberculosis*), *M. bovis*, *M. africanum*, *M. microti*, *M. canetti*, *M. caprae* and *M. pinnipedi* [15]. Each of the closely related members of the *M. tuberculosis* complex (MTBC) can cause tuberculosis in humans; however, most cases worldwide are caused by *M. tuberculosis*. The other primarily human pathogens, *Mycobacterium africanum* and *Mycobacterium canettii*, are seen less often and are more restricted to patients from sub-Saharan Africa [15]. *Mycobacterium tuberculosis* is spread by small airborne droplets, called droplet nuclei, generated by the coughing, sneezing, talking, or singing of a person with pulmonary or laryngeal tuberculosis. These minuscule droplets can remain airborne for minutes to hours after expectoration [16]

#### **2.1.2. Pathophysiology**

Once inhaled, the infectious droplets settle throughout the airways. The majority of the bacilli are trapped in the upper parts of the airways where the mucus-secreting goblet cells exist. The mucus produced catches foreign substances, and the cilia on the surface of the cells constantly beat the mucus and its entrapped particles upward for removal [17]. This system provides the body with an initial physical defense that prevents infection in most persons exposed to tuberculosis [18].

Bacteria in droplets that bypass the mucociliary system and reach the alveoli, are quickly surrounded and engulfed by alveolar macrophages [17, 19]. These macrophages provide an opportunity for the body to destroy the invading Mycobacteria and prevent infection [20]. The mycobacterial lipoarabinomannan is a key ligand for a macrophage receptor [21]. The complement protein C3 binds to the cell wall and enhances recognition of the Mycobacteria by macrophages [22]. The subsequent phagocytosis by macrophages initiates a cascade of events that results in either successful control of the infection, followed by latent tuberculosis, or progression to active disease, called primary progressive tuberculosis [17]. The outcome is essentially determined by the quality of the host defenses and the balance that occurs between host defenses and the invading Mycobacteria [20, 23].

After being ingested by macrophages, the Mycobacteria continue to multiply slowly [17], with bacterial cell division occurring every 25 to 32 hours [14, 19]. Regardless of whether the infection becomes controlled or progresses, initial development involves production of proteolytic enzymes and cytokines by macrophages in an attempt to degrade the bacteria [20, 21]. Released cytokines attract T lymphocytes to the site, the cells that constitute cell-mediated immunity. Macrophages then present mycobacterial antigens on their surface to the T cells [20]. This initial immune process continues for 2 to 12 weeks; the microorganisms continue to grow until they reach sufficient numbers to fully elicit the cell-mediated immune response, which can be detected by a skin test [14, 17, 20].

For persons with intact cell-mediated immunity, the next defensive step is formation of granulomas around the *M tuberculosis* organisms [24]. These nodular-type lesions form an accumulation of activated T lymphocytes and macrophages, which creates a micro-environment that limits replication and the spread of the Mycobacteria [17, 21]. This environment destroys

macrophages and produces early solid necrosis at the center of the lesion; however, the bacilli are able to adapt and survive [25]. In fact, *M tuberculosis* organisms can change their phenotypic expression, such as protein regulation, to enhance survival [22]. By 2 or 3 weeks, the necrotic environment resembles soft cheese, often referred to as caseous necrosis, and is characterized by low oxygen levels, low pH, and limited nutrients. This condition restricts further growth and establishes latency. Lesions in persons with an adequate immune system generally undergo fibrosis and calcification, successfully controlling the infection so that the bacilli are contained in the dormant, healed lesions [25]. The lesions in persons with less effective immune systems progress to primary progressive tuberculosis [14, 17, 22, 25].

### **2.1.3. Tuberculosis disease**

*Mycobacterium tuberculosis* organisms can be enclosed, as previously described, but are difficult to completely eliminate [23]. Persons with latent tuberculosis have no signs or symptoms of the disease, do not feel sick, and are not infectious [26]. However, viable bacilli can persist in the necrotic material for years or even a lifetime [18], and if the immune system later becomes compromised, as it does in many critically ill patients, the disease can be reactivated.

The primary lesion may enlarge; pleural effusion is a distinguishing finding. The effusion may remain small and resolve spontaneously, or it may become large enough to induce symptoms such as fever, pleuritic chest pain, and dyspnea [19]. Manifestations often include progressive fatigue, malaise, weight loss, and a low-grade fever accompanied by chills and night sweats [27].

Although the pulmonary system is the most common location for tuberculosis, extra-pulmonary disease occurs in more than 20% of immunocompetent patients, and the risk for extra-pulmonary disease increases with immunosuppression [28]. The most serious location is the central nervous system, where infection may result in meningitis or space-occupying tuberculomas. Another fatal form of extra-pulmonary tuberculosis is infection of the bloodstream by *Mycobacteria*; this



form of the disease is called disseminated or miliary tuberculosis. The bacilli can then spread throughout the body, leading to multi-organ involvement [29]. Lymphatic tuberculosis is the most common extra-pulmonary tuberculosis, and cervical adenopathy occurs most often. Other possible locations include bones, joints, pleura, and genitourinary system [28].

## **2.2.Epidemiological aspect of tuberculosis**

Tuberculosis is a disease of poverty that thrives where social and economic determinants of ill health prevail, and it affects mostly young adults in their most productive years; 95% of TB deaths are in the developing world [12].

There were an estimated 12 million prevalent cases (range, 10 to 13 million) of TB in 2012 worldwide. In 2012, an estimated 8.7 million incident cases of TB (range, 8.3 million to 9 million) globally, equivalent to 125 cases per 100,000 population. Most of the estimated cases in 2011 occurred in Asia (59%) and Africa (26%). In 2011 WHO reported that Rwanda had 14,000 (6,600-21,000) tuberculosis cases with prevalence rate of 128 (62-197).

Of the 8.7 million incident cases in 2012, 1.1 million (1.0 to 1.2 million or 12 to 14%) were among people living with HIV. The proportion of TB cases coinfecting with HIV was the highest in the African Region countries; overall, 39% of TB cases were estimated to be coinfecting with HIV in this region. The African Region accounts for almost 80% of HIV-positive TB cases worldwide. Tuberculosis incidence rate in HIV positive in Rwanda were estimated to be 34 (30-38) in 2011 [30].

## **2.3.Performance of tuberculosis diagnosis methods**

### **2.3.1. Microscopy**

The sensitivity of the direct smear assay has been found to be dependent on staff that has been well trained so that sufficient time is spent on preparing, staining, and reading each smear, with a well-functioning external quality assurance (EQA) program in place. In some studies,

microscopy has been reported to have greater than 80% sensitivity for identifying cases of pulmonary tuberculosis however, in other reports; the sensitivity of the test has been relatively low and variable (range 20–60%)[31] though sputum smear microscopy method could detect around 70% of pulmonary tuberculosis cases in optimal conditions [5].

Several studies with culture as the reference standard indicated that the sensitivity of conventional microscopy ranged from 32 to 94% and that the sensitivity of fluorescence microscopy ranged from 52 to 97%, with the fluorescent method being on average 10% more sensitive than light microscopy [4]. Because fluorochrome-stained smears can be examined at lower magnifications, it takes less time to examine these smears than to examine smears stained with ZN stain and still results in a higher sensitivity and a similar specificity.

Several LED technologies are now available offering low-cost, battery-operated stand-alone microscopes or add-on components and for the most, sensitivity and specificity has been demonstrated to be equivalent to standard FM [32-37] and sensitivity superior to ZN microscopy [33, 35, 38-40]. These findings were also confirmed for HIV-infected patients[41, 42]; however, three studies reported equal sensitivity [43] or lower specificities [39, 44, 45] for LED FM compared with ZN. In these studies, readers had no previous experience with fluorescence microscopy, which is the most likely explanation for performance differences compared with other studies. This highlights the importance of adapting training intensity according to the level of operator proficiency.

Fluorescence microscopy (FM) saves up to 75% in reading time, and requires simpler staining methods [46]. Implementation in resource-limited settings has been minimal due to high unit costs, maintenance needs, the fragility of mercury vapor bulbs, and technician reluctance to work in a darkened room [47]. The use of LED as an alternative light source has helped overcome these hurdles [48].

### **2.3.2. Mycobacterial culture and drug susceptibility testing**

Culture is more sensitive than AFB smear microscopy for the detection of *M. tuberculosis*: while microscopy requires approximately 5,000 to 10,000 AFB/ml of sputum for detection, culture can detect as few as 10 to 100 viable bacteria/ml [49]. Although culture is not routinely performed in resource poor settings except for purposes of surveillance, treatment failure, or detection of drug resistance, culture is increasingly recognized as being critically important in detecting TB in HIV-positive individuals who often have low bacillary loads in sputum specimens [50].

The most widely available mycobacterial culture system has been egg-based Löwenstein-Jensen (LJ) solid medium[51]. Although relatively inexpensive and simple to perform, its major drawback is the time required for the development of a positive culture, taking up to 6 weeks for paucibacillary specimens. Drug-susceptibility testing (DST) has been typically performed on solid media, the most common method being the proportion method on LJ media [51]. Similarly, the long time to availability of results, up to 2 months or more from the time of specimen collection to reporting of DST results, is a major shortcoming. Several non-radiometric liquid culture and DST methods have also been introduced and continue to be evaluated in various laboratories throughout the world. Among these, the Mycobacterial Growth Indicator Tube (MGIT) system that is based on detection of bacterial consumption of oxygen in the culture medium with a fluorescent sensor has been widely evaluated. These studies have repeatedly shown that MGIT culture significantly decreases the time to detection and increases the recovery of mycobacteria from clinical specimens compared with solid media [52-60]. Automated liquid culture systems can cut the time to detection of Mycobacteria by between 5 and 12 days and decrease the number of false-negative results by 50% [61]. Additionally, the accuracy of liquid DST methods in the detection of isoniazid (INH), rifampicin (RIF), streptomycin, and ethambutol resistance has been shown to be comparable to “gold standard” conventional methods [62-66]. A common concern regarding the implementation of liquid culture is that it is

more prone to contamination with environmental bacteria and those that are commonly present as normal flora in clinical specimens. The comparisons of contamination rates have ranged from < 1 to 17% and from 3 to 19% for solid and liquid media, respectively[61].

Liquid culture systems have shown 10 to 15% more sensitivity than LJ; the median time to detection was 9 days compared with 28 days for LJ, and, importantly, initially high contamination rates of over 20% decreased to 7 and 12% over time [66].

### **2.3.3. Nucleic acid amplification test (NAAT)**

To date, only two molecular technologies have been endorsed by WHO for use in low and middle income countries: line probe assays for the rapid detection of MDR-TB and cartridge-based nucleic acid amplification test (NAAT) for TB and multi-drug resistant tuberculosis (MDR-TB) detection.

#### **2.3.3.1. Line Probe Assays for MDR-TB**

In 2008 WHO endorsed the use of line probe assays (LPAs) for the rapid detection of MDR-TB [67]. Currently, there is only one LPA for molecular TB drug resistance globally available, the GenoType MTBDRplus® produced by Hain Life science. The assay includes *rpoB* probes for RIF resistance, *katG* probes for high-level INH resistance, and *inhA* probes for determination of low-level INH resistance. Different laboratory-based studies found that the pooled sensitivity and specificity estimates for RIF resistance were 98 and 99%, respectively and highly consistent among all patient subgroups and specimen types. The sensitivity estimate for INH resistance was lower (84%) and more varied, whereas the specificity estimate remained high (99%). All studies found a high level of interpretable results, although this decreased with decreasing AFB smear grade [68]. The LPAs are substantially more complex to perform than conventional methods for DST and require skilled and well-trained laboratory personnel, as well as additional laboratory space in which to perform the assays to reduce the risk of amplicon contamination. However,

they offer significant advantage over current methods in terms of simultaneous confirmation of MTB complex and detection of resistance to RIF and INH in 1 to 2 days directly from smear-positive sputum specimens and a higher proportion of interpretable results than liquid culture and DST.

An updated version of the MTBDRplus was introduced by Hain in 2011 that has increased sensitivity for TB detection and may be performed directly on AFB smear-negative specimens. In an assessment of this assay in Moldova, the sensitivity and specificity for TB detection in 104 AFB smear-negative, culture-positive respiratory specimens were 80 and 99%, respectively, whereas those for RIF and INH resistance were 94 and 96%, respectively [69]. Another study from a high-volume public health laboratory in South Africa compared the second-generation MTBDRplus with the Xpert MTB/RIF (Cepheid, Sunnyvale, CA) test [70]. In this study, the results of the two molecular assays were compared with phenotypic culture and DST. The sensitivities of the Hain LPA and Xpert for detecting culture-positive TB were 72 and 71%, respectively, and sensitivities for RIF resistance were 99 and 98%, respectively. Detection rates for AFB smear-negative, culture-positive specimens were 58% for the Hain LPA and 57% for Xpert, somewhat lower than that found in the study in Moldova. This most recent Hain LPA version has not yet been endorsed by WHO for testing on smear-negative specimens.

#### **2.3.3.2. Cartridge-Based NAAT**

The Xpert MTB/RIF assay is the first of a new generation of macro-fluidics based molecular tests that integrates sample processing, amplification, and detection in a closed, single-use cartridge, thereby minimizing DNA contamination risk and biosafety requirements [71]. The real-time PCR-based assay targets the 81-base-pair region of the RNA polymerase (*rpoB*) gene to detect MTB and RIF resistance, has a limit of detection of 131 CFU/ml in spiked sputum, a

high analytical specificity against non-tuberculosis Mycobacteria (NTM) species [72, 73], and provides the end user with a semi-quantitative readout [74].

In 2010, WHO recommended that Xpert be used at district and subdistrict levels as the initial diagnostic test in individuals suspected of having MDR-TB or HIV-associated TB and be considered as a follow-on test for smear-negative patients in other settings [7]. The clinical accuracy data reviewed as a basis for this recommendation consisted of a published multicenter study [75] and, at the time, largely unpublished data of single-center studies [76-80] and a multicenter study conducted in peripheral laboratories [81].

Since then, there has been a proliferation of reports on the clinical performance of this technology. In a Cochrane review, the pooled median sensitivity and specificity of Xpert compared with culture were 88% (95% CI 83 to 92) and 98% (97 to 99), respectively, with a sensitivity in smear-positive cases of 98% (97 to 99) and 68% (60 to 75) in smear-negative cases [9]. In HIV-positive TB cases, the sensitivity appeared lower than in HIV-negative patients (76% [63, 85] compared with 89% [81, 94] pooled median sensitivity), whereas there was little difference in specificity [9]. Studies published in 2012 all report a high Xpert sensitivity in HIV-positive patients [82-85]. Xpert correctly identified RIF resistance with a pooled median sensitivity of 94% (87, 97) and correctly excluded RIF resistance in 98% (97, 99) [9]. Across all these studies, specificity was high and Xpert offered significant advantages over smear microscopy in terms of sensitivity and over culture in terms of time to detection. Moreover Xpert in a variety of extra-pulmonary data show a rapid diagnosis in ~ 50 to 80% of extra-pulmonary cases depending on specimen type and processing method [86]; several studies have looked at the diagnostic accuracy of Xpert in a variety of extra-pulmonary samples, including biopsies and aspirates of tissue samples and lymph nodes; body fluids including pleural, pericardial, and cerebrospinal fluid; pus; gastric lavages; urine; and stool [76, 79, 87-94].

## **2.4.Treatment**

The goals of TB treatment are to ensure cure without relapse, to prevent death, to stop transmission, and to prevent the emergence of drug resistance. Almost all recommended treatment regimens have two phases [95, 96]. There is an initial intensive phase in which at least two bactericidal drugs, isoniazid and rifampicin. Pyrazinamide given in the initial intensive phase allows the duration of treatment to be reduced from 9 to 6 months[97]. The addition of Ethambutol benefits the regimen when initial drug resistance may be present or the burden of organisms is high. The continuation phase eliminates most residual bacilli and reduces numbers of failures and relapses. At the start of the continuation phase there are low numbers of bacilli and less chance that drug-resistant mutants will be selected, and therefore fewer drugs (RIF and INH) are needed [98, 99].

## **2.5.Control**

### **2.5.1. DOTS strategy**

To control tuberculosis, WHO and IUATLD recommend the DOTS strategy [10], which has five elements: political commitment, diagnosis primarily by sputum-smear microscopy among patients attending health facilities, short-course treatment with effective case management (i.e. direct observation), regular drug supply, and systematic monitoring to assess outcomes of every patient started on treatment. Standard short-course regimens can cure more than 95% of cases of new, drug-susceptible tuberculosis. The international targets for tuberculosis control by 2005 was to detect 70% of new pulmonary smear-positive cases annually and to treat 85% of detected cases successfully [100] which many developing countries has not yet reached up to date.

### **2.5.2. BCG vaccination**

Randomized and case-control trials have shown consistently high protective efficacy of BCG against serious forms of disease in children i.e. meningitis and miliary tuberculosis, but variable

efficacy against pulmonary tuberculosis in adults [101]. Thus, in high-prevalence areas, vaccination is recommended for children at birth or at first contact with health services, except for children with symptomatic HIV infection [102]. Even with high coverage, BCG has not had any substantial effect on transmission or incidence, because its main action is to prevent serious disease in children.

### **2.5.3. Treatment of latent tuberculosis infection**

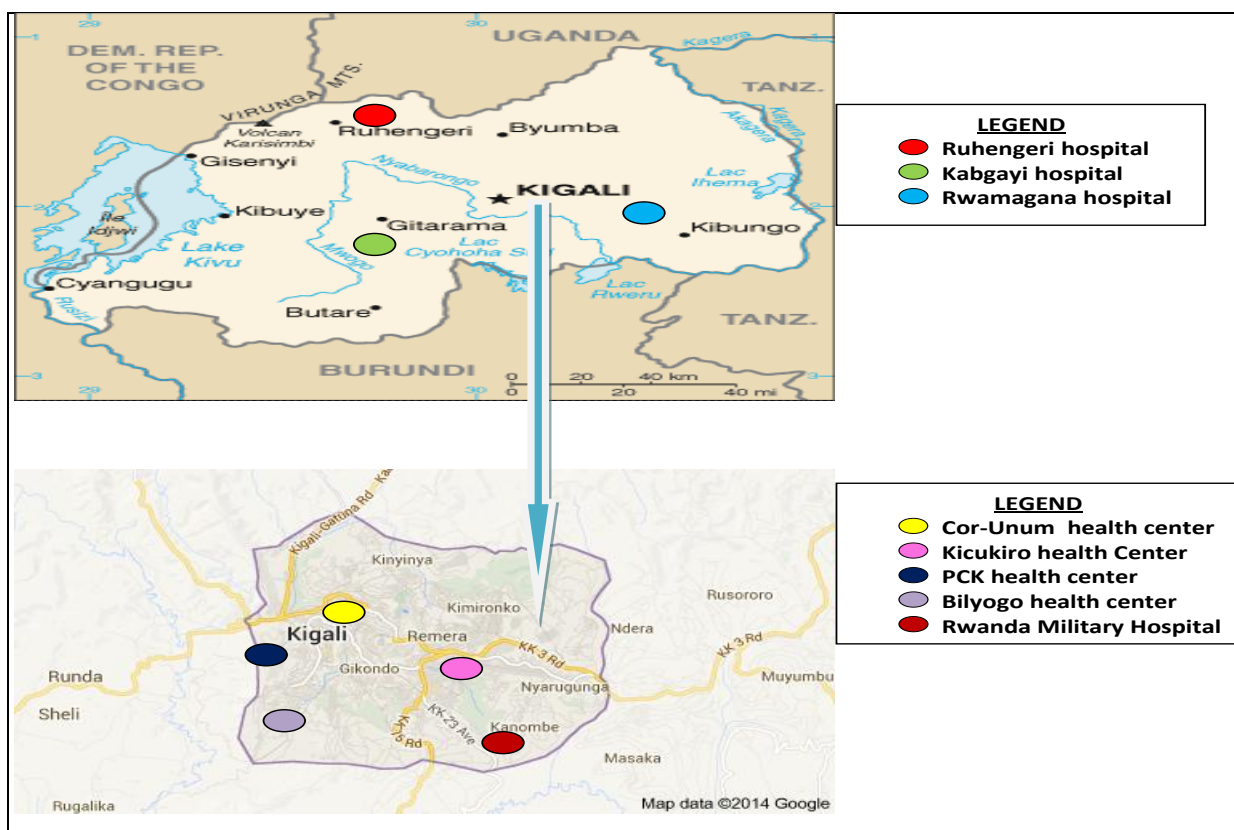
Treatment of latent infection has generally consisted of daily administration of isoniazid for 6—12 months. Such treatment is 60—90% effective in reducing the risk of progression from tuberculosis infection to disease [103]. HIV-infected, tuberculin-positive individuals can benefit greatly from treatment of latent tuberculosis infection, if practical aspects of program administration can be addressed. Contacts of active cases (especially children), recent converters to tuberculin skin test positivity, and selected individuals at high risk of disease can also benefit [104].



### **3.0.METHODOLOGY**

#### **3.1. Study area**

The study was conducted at four intermediate and four peripheral health facility laboratories across the country. One of each type of health facility was using ZN staining while the other was using LED-FM for diagnosis of pulmonary tuberculosis. Selected peripheral health facilities with LED-FM were Kicukiro and Bilyogo; those with ZN method were PCK and Cor-Unum. Intermediate health facilities with LED-FM were Kabgayi and Rwamagana; those with ZN method were Ruhengeri and Rwanda Military Hospital. These health facility laboratories had recorded the highest number of pulmonary tuberculosis cases suspect in the 2013 records. Rwamagana, Kabgayi and Ruhengeri hospitals are located in the Eastern, Southern and Northern provinces respectively. Rwanda Military hospital and the four peripheral health centers (Cor-Unum, Bilyogo, Kicukiro and PCK) are located in Kigali city. Intermediate health facility laboratories or District hospital laboratories have an average of six qualified laboratory technologists supervised, trained and mentored by the NRL. Peripheral health facility laboratories or health center laboratories have an average of two laboratory technicians supervised, trained and mentored by intermediate health facility laboratories. The ZN and LED-FM using by health facility laboratories record an average of 625 and 400 TB suspect cases respectively per year. Rechecking of sputum smear microscopy is performed quarterly and the proficiency panel testing is done once per year by the NRL. The smear positivity rate for the year 2013 was an average of 12% and 5% by LED-FM and ZN from health facility laboratories respectively.



**Figure 1:** Map of Rwanda with location of health facility assessed in the study

### 3.2. Study design

This was a cross-sectional study conducted between April and August, 2014.

### 3.3. Study population

The study targeted sputum specimens and pre-prepared stained sputum smear collected from participants with suspected pulmonary TB visiting any of the eight health facility within the period of study.

### 3.4. Study sample and sampling strategy

The minimum sample size was 646 sputum specimens from the eight health facilities; each providing a number of specimens according to the number of TB suspected participants received during the sample collection period.

The formula below was used to calculate the sample size [105, 106]

Sample size (n) based on sensitivity: 
$$n = \frac{z_{\alpha/2}^2 \times S_N(1-S_N)}{L^2 \times P}$$

Sample size (n) based on specificity: 
$$n = \frac{z_{\alpha/2}^2 \times S_P(1-S_P)}{L^2 \times P}$$

**Description:**

**n** = required sample size for this study.

**P**= Estimated prevalence of pulmonary TB in TB suspect attending health facilities =0.5

**S<sub>N</sub>**= anticipated sensitivity = 0.7 [107]

**S<sub>P</sub>**= anticipated specificity (=0.95) [4]

**α**= size of the critical region (1 – α is the confidence level) = 0.05

**z<sub>α/2</sub><sup>2</sup>**= standard normal deviate corresponding to the specified size of the critical region (α) =1.96,

and

**L**= absolute precision desired on either side (half-width of the confidence interval) of sensitivity or specificity=0.05

The sample size calculated based on the sensitivity of ideal smear microscopy results is the largest and will be used in this study

$$n \text{ based on } S_N = \frac{1.96^2 \times 0.7(1 - 0.7)}{0.05^2 \times 0.5} = \frac{0.806736}{0.00125} = 645.38 \cong \mathbf{646}$$

The study consecutively recruited consented TB suspected participants attending health facilities till the required sample size was achieved.

**3.4.1. Inclusion criteria**

All suspected TB patients, aged 18 years and above who gave informed consent to participate in the study and give sputum specimens were recruited.

**3.4.2. Exclusion criteria**

All TB patients under treatment or TB suspects who were unable to give sputum specimens were excluded from the study.

### **3.5.Laboratory procedures**

#### **3.5.1. Specimens collection and handling**

Three to five milliliter of morning sputum specimen were collected from each eligible participants in a clean plastic container with wide-mouthed, screw-capped and leak proof. A direct sputum smear was prepared, stained and examined by laboratory technicians at health facility laboratory. The remaining sputum specimens and the examined corresponding sputum smear were immediately shipped to the tuberculosis laboratory of NRL. Sputum specimens not shipped immediately were refrigerated (4 to 8°C). All sputum specimens collected were transported in a cool box (4-8°C) and were processed on the same day at NRL TB laboratory. No sputum preservative such as CPC-NaCl was used.

#### **3.5.2. Specimen processing and laboratory testing**

At NRL, sputum specimens were recorded and processed using N-Acetyl-Cystein Sodium hydroxide procedure (Appendix 7). The pellet obtained from processed specimens was used to inoculate MGIT (0.5ml) and 2 LJ tubes (150-200µl) respectively as per the procedure indicated above. The remaining pellet was used to prepare two smears and to run Xpert MTB/RIF test (Appendix 6). The two smears prepared from pellet were stained using auramine and ZN for LED-FM and light microscopy examination respectively (Appendix 8 & 9). The smears collected from health facility laboratory were reexamined at NRL to confirm the result.

The inoculated MGIT were automatically incubated in a BD BACTEC 960 machine for up to 42 days to confirm negative culture results while the 2 LJ tubes were incubated in manual incubator at 37°C and weekly readings made for up to the eighth week to confirm culture negative results.

Species identification and drug susceptibility testing was performed for all positive cultures. Species identification was done using SD-Bioline, an immunochromatographic test which detects specific prominent antigens MPT64 produced by *Mycobacterium tuberculosis* complex during growth (Appendix 10). The drug susceptibility testing for rifampicin was carried out by Xpert MTB/RIF test (Appendix 6).

### **3.5.3. Quality control**

For QC, AFB smear positive control was run with each batch of testing of specimens using ZN and Auramine staining. The standard *Mycobacterium tuberculosis* strain (HRV37) was used to control the ability of culture media to support growth for each lot of LJ media used. Standard operating procedures were followed for all laboratory testing. All reagents preparation and testing procedure were done following the kits and equipment manufacturer's instructions.

### **3.6.Data collection and management**

Informed consent was sought from all eligible participants and sputum samples collected. The patient's laboratory request form was retrieved from respective health facilities and data abstraction done and the information entered in a data collection tool. The information abstracted from the laboratory request form included gender, age, residence, signs and symptoms from clinical examination, results of sputum smear microscopy at health facility and HIV sero-status. In addition data obtained from further testing were recorded from specimen processing register at the NRL. From data collection tool, data were entered in a Microsoft Excel databases; followed by data cleaning for errors. Data collected were kept in lockable cabinets where only the researcher had access to maintain confidentiality. Information stored in Microsoft excel based databases was protected from unauthorized individuals with the use of a password. All records were identified by study identification numbers to maintain confidentiality.

### 3.7.Data analysis and presentation

Reexamination of smear from health facility at NRL, results of concentrated smear and Xpert MTB/RIF test provided preliminary results for treatment of pulmonary tuberculosis cases missed by health facility laboratories. The final results were provided by culture.

Sensitivity, specificity and predictive values were calculated using the following below formula[2]:

$$Se = \frac{TP}{TP + FN}; Sp = \frac{TN}{TN + FP}; PPV = \frac{TP}{TP + FP}; NPV = \frac{TN}{TN + FN}$$

$$95\%, \text{ C.I } p - 1.96\sqrt{\frac{p(1-p)}{n}} \leq \pi \ll p + 1.96\sqrt{\frac{p(1-p)}{n}}$$

Where Se: sensitivity, Sp: specificity, TP: True positive results; TN: True negative results; FN: False negative results; FP: False positive results. These results were defined based on ZN or LED-FM microscopy results versus culture as gold standard. The increment yield of Xpert MTB RIF test was obtained based on its sensitivity against sputum smear microscopy compared to the mycobacterial culture as gold standard. Results were presented in tables.

### 3.8.Ethical consideration

This study obtained ethical approval from the Rwanda National Ethical Committee and Kenyatta National Hospital/University of Nairobi Ethic and Research committee before execution (Appendix 11, 12 and 13). Patients were informed about their results through their respective health facility. A detailed report regarding the outcome results of the study was sent to the NRL and NTP, to inform about the accuracy of currently in use TB diagnostic methods and the incremental yield of Xpert MTB/RIF in intermediate and peripheral health facilities.

## **4.0.RESULTS**

### **4.1.Socio-demographic characteristics of participants and MTB positivity by methods**

The study enrolled 648 participants, of whom 402 (62%) were males and 246 (38%) were females. The participants' age ranged from 18 to 94 years, with a mean of 40.5, standard deviation of 15.7 years and the mode age was between 28 and 37 years. More than half (64%) of the study population were from Bilyogo (24%), Cor-Unum (22%), and Kabgayi (18%) whereas the remaining 36 % were from PCK (12%), Kicukiro (8%), Ruhengeri (7%), RMH (6%) and Rwamagana (3%) health facility laboratories respectively. Among 648, 428 (66%) and 220 (34%) were from peripheral and intermediate health facility respectively, 344 (53%) and 304 (47%) participants were from LED-FM and ZN-using health facility laboratories respectively. Of the 648 participants, 179 were HIV positive and 469 were HIV negative. The direct smear microscopy at health facility laboratories showed 51 to be AFB positive and 597 to be AFB negative (positivity rate of 7.8%), reexamination of these smear at NRL showed that 56 were AFB positive and the smear prepared after specimen processing and concentration revealed 60 to be AFB positive. The Xpert MTB/RIF test showed 82 specimens to be MTB positive (positivity rate 12.6%) with 7 MTB positive being rifampicin resistant. Three specimens did not get a final results on the Xpert MTB/RIF test (error) and one MTB positive did not get a final result of rifampicin test (indeterminate). Of the 648 specimens, mycobacterial culture yielded 97 MTB positive growth (positivity rate 14.9%), 22 (3.3%) non tuberculosis mycobacterium (NTM) and 23 (3.5%) cultures were contaminated (Table 1).

**Table 1:** Demographic distribution of participants and MTB positivity rate by methods

|   |                         | <b>Participants</b> |                     | <b>Percentage</b>   |              |
|---|-------------------------|---------------------|---------------------|---------------------|--------------|
| <b>Gender</b>   | Male                    | 402                 |                     | 62                  |              |
|   | Female                  | 246                 |                     | 38                  |              |
| <b>Age bracket (years)</b>                                  | 18-27                   | 150                 |                     | 23.2                |              |
|   | 28-37                   | 178                 |                     | 27.4                |              |
|   | 38-47                   | 123                 |                     | 19                  |              |
|   | 48-57                   | 87                  |                     | 13.4                |              |
|   | 58-67                   | 64                  |                     | 9.8                 |              |
|   | 68 and above            | 46                  |                     | 7.2                 |              |
| <b>Health facility laboratories</b>                         | Bilyogo                 | 155                 |                     | 24                  |              |
|   | Cor-Unum                | 142                 |                     | 22                  |              |
|   | Kabgayi                 | 118                 |                     | 18                  |              |
|   | PCK                     | 76                  |                     | 12                  |              |
|   | Kicukiro                | 54                  |                     | 8                   |              |
|   | Ruhengeri               | 48                  |                     | 7                   |              |
|   | RMH                     | 38                  |                     | 6                   |              |
|   | Rwamagana               | 17                  |                     | 3                   |              |
| <b>HF laboratory by techniques</b>                          | LED-FM peripheral lab   | 209                 |                     | 32.3                |              |
|   | ZN peripheral lab       | 218                 |                     | 33.6                |              |
|   | LED-FM intermediate lab | 135                 |                     | 20.8                |              |
|   | ZN intermediate lab     | 86                  |                     | 13.3                |              |
| <b>HIV-sero status</b>                                      |                         | <b>Positive</b>     |                     | <b>Negative</b>     |              |
|   |                         | 179                 |                     | 469                 |              |
| <b>Smear results at health facility</b>                     |                         | <b>AFB positive</b> |                     | <b>AFB negative</b> |              |
|   |                         | 51                  |                     | 597                 |              |
| <b>Smear reexamination at NRL</b>                           |                         | 56                  |                     | 592                 |              |
| <b>Smear prepared after specimen processing for culture</b> |                         | 60                  |                     | 588                 |              |
| <b>Xpert MTB/RIF test</b>                                   |                         | <b>MTB negative</b> | <b>MTB positive</b> | <b>Error</b>        |              |
|   |                         | 563                 | 82                  | 3                   |              |
| <b>Mycobacterial culture</b>                                |                         | <b>Negative</b>     | <b>Positive</b>     | <b>NTM</b>          | <b>Cont.</b> |
|   |                         | 506                 | 97                  | 22                  | 23           |

*NTM: Non-Tuberculous Mycobacteria; Cont.: Contaminated culture*



#### **4.2. Mycobacterial positivity by different methods used in our study population**

Mycobacterial cultures (LJ and MGIT) yielded 97 (14.9%) growth of *Mycobacterium tuberculosis* complex (MTB) while 506 (78%) did not grow any organism.

Of the 97 culture positive, 72 (74%) were males (prevalence in male 18%) and 25 (26%) were females (prevalence in female 10%), 28 (28.8%) were HIV sero-positive, 61 (62.8%) were from ZN-using health facility laboratories and 36 (37.2%) were from LED-FM using health facility laboratories. Among 648 participants, the direct smear examination at health facility laboratories showed that 51 (7.8%) were AFB positive whereas 597 (92.2%) were AFB negative. Of the 51 AFB positive 38 (75.5%) and 13 (24.5%) were males and females respectively (male/female ratio = 3:1). Among the 648 participants, 179 (27.6%) and 469 (72.4%) were HIV positive and HIV negative respectively (HIV-negative/HIV-positive ratio = 2.6:1). Among the 179 HIV-positive, 105 were males and 74 were females, 11 (21.5%) were AFB positive and 168 (78.5%) were AFB negative by direct smear examination at health facility laboratories.

Of the 648 participants, Xpert MTB/RIF test detected 82 (12.6%) *Mycobacterium tuberculosis* complex, 563 (87.4%) were MTBC negative and 3 did not get a final results (error). Of the 82 MTB positive by Xpert MTB/RIF test 74 were rifampicin susceptible, 7 were rifampicin resistant and one was rifampicin invalid.

**Table 2:** Mycobacterial positivity by different methods used in the study population

|                         |              | Mycobacterial culture                |                      |                    |                  |       |
|-------------------------|--------------|--------------------------------------|----------------------|--------------------|------------------|-------|
|                         |              | MTB positive                         | MTB negative         | NTM                | Contaminated     |       |
| <b>Gender</b>           | Male         | 72                                   | 303                  | 10                 | 17               |       |
|                         | Female       | 25                                   | 203                  | 12                 | 6                |       |
| <b>HIV status</b>       | Positive     | 28                                   | 136                  | 7                  | 8                |       |
|                         | Negative     | 69                                   | 370                  | 15                 | 15               |       |
|                         |              | Xpert MTB/RIF test                   |                      |                    |                  |       |
|                         |              | Negative                             | MTB, RIF susceptible | MTB, RIF resistant | MTB, RIF invalid | Error |
| <b>Gender</b>           | Male         | 339                                  | 54                   | 5                  | 1                | 3     |
|                         | Female       | 224                                  | 20                   | 2                  |                  |       |
| <b>HIV-sero status</b>  | Positive     | 154                                  | 20                   | 2                  | 1                | 2     |
|                         | Negative     | 409                                  | 54                   | 5                  |                  | 1     |
|                         |              | Direct smear at health facility labs |                      |                    |                  |       |
|                         |              | AFB positive                         | AFB negative         | Positivity rate    |                  |       |
| <b>Gender</b>           | Male         | 38                                   | 364                  | 9.4%               |                  |       |
|                         | Female       | 13                                   | 233                  | 5.2%               |                  |       |
| <b>HIV-sero status</b>  | Positive     | 11                                   | 168                  | 6.1%               |                  |       |
|                         | Negative     | 40                                   | 429                  | 8.5%               |                  |       |
| <b>ZN technique</b>     | Intermediate | 7                                    | 79                   | 8.1%               |                  |       |
|                         | Peripheral   | 27                                   | 191                  | 12%                |                  |       |
| <b>LED-FM technique</b> | Intermediate | 6                                    | 129                  | 4.4%               |                  |       |
|                         | Peripheral   | 11                                   | 198                  | 5.2%               |                  |       |

#### 4.3. Performance of sputum smear microscopy at health facility laboratories

##### 4.3.1. Direct smear at health facility lab versus the concentrated smear

Direct smear prepared at health facility laboratory were re-stained and reexamined at the NRL. Of the 597 AFB negative by health facility laboratories, six were revealed to be AFB positive by re-examination and one AFB positive smear by health facility laboratories was revealed to be AFB negative by reexamination. Of the 597 AFB negative specimens at health facility laboratories, 12 smears prepared after specimen processing were AFB positive. In contrast, three processed smears were AFB negative while they were initially AFB positive by health facility laboratories. Among the 12 AFB positive smear prepared after processing, 9 were AFB negative by reexamination of direct smear at NRL and 3 smears which initially were AFB positive by reexamination turned out to be AFB negative on the smear prepared after specimen processing.

**Table 3:** Direct microscopy versus smear prepared after specimen processing

|                               | <b>Smear prepared after specimen processing</b> |            |        |        |        |
|-------------------------------|---|------------|--------|--------|--------|
| <b>Direct smear at HF lab</b> | AFB negative                                    | AFB scanty | AFB 1+ | AFB 2+ | AFB 3+ |
| AFB negative                  | 585   | 9          |        | 2      | 1      |
| AFB scanty                    | 3   | 3          | 3      | 2      | 3      |
| AFB 1+                        |   |            | 2      | 5      | 7      |
| AFB 2+                        |   | 2          | 2      | 2      | 5      |
| AFB 3+                        |   |            | 1      |        | 11     |
|                               | <b>Direct smear reexamination at NRL</b>        |            |        |        |        |
| AFB negative                  | 586   | 6          |        |        |        |
| AFB scanty                    | 3   | 6          | 4      | 3      | 1      |
| AFB 1+                        |   | 1          | 3      | 7      | 7      |
| AFB 2+                        |   |            | 1      | 1      | 10     |
| AFB 3+                        |   |            |        |        | 9      |

#### **4.3.2. Direct smear microscopy versus Xpert MTB/RIF test**

Of the 82 MTB positive by Xpert MTB/RIF, 36 (44%) were negative by direct smear microscopy while four AFB positive specimen were not detected by Xpert MTB/RIF test. Among the 36 AFB negative but MTB positive by Xpert MTB/RIF, 13 (36%) were from HIV sero-positive patients. The direct smear microscopy was positive for AFB in 11 cases out of 24 HIV positive detected MTB positive by Xpert MTB/RIF test. Indeed of the 7 rifampicin resistant MTB detected by Xpert MTB/RIF, only two specimens were AFB positive by direct smear microscopy. Among 36 MTB positive by Xpert MTB/RIF and AFB negative by direct smear microscopy, 22 were from ZN-using health facility laboratories and 14 were from LED-FM using health facility laboratories, 29 were from peripheral health facility laboratories and only seven were from intermediate health facility laboratories

**Table 4:** Direct smear microscopy versus Xpert MTB/RIF test

| Microscopy                          |                     | Xpert MTB/RIF test |                               |                             |                           |       |
|-------------------------------------|---------------------|--------------------|-------------------------------|-----------------------------|---------------------------|-------|
|                                     |                     | MTB negative       | MTB positive, RIF susceptible | MTB positive, RIF resistant | MTB positive, RIF invalid | Error |
| AFB positive                        | HIV positive        | 1                  | 10                            |                             |                           |       |
|                                     | HIV negative        | 3                  | 34                            | 2                           |                           | 1     |
| AFB negative                        | HIV positive        | 153                | 10                            | 2                           | 1                         | 2     |
|                                     | HIV negative        | 406                | 20                            | 3                           |                           |       |
| <b>Health facility laboratories</b> |                     |                    |                               |                             |                           |       |
| AFB positive                        | ZN intermediate     |                    | 7                             |                             |                           | 1     |
|                                     | ZN peripheral       | 3                  | 23                            | 1                           |                           |       |
|                                     | LED-FM intermediate |                    | 5                             |                             |                           |       |
|                                     | LED-FM peripheral   | 1                  | 9                             | 1                           |                           |       |
| AFB negative                        | ZN intermediate     | 75                 | 3                             | 1                           |                           |       |
|                                     | ZN peripheral       | 173                | 14                            | 3                           | 1                         |       |
|                                     | LED-FM intermediate | 126                | 3                             |                             |                           |       |
|                                     | LED-FM peripheral   | 185                | 10                            | 1                           |                           | 2     |

**4.3.3. Direct smear microscopy versus mycobacterial culture**

Of the 97 MTB culture positive, 47 (48%) were AFB negative and all NTM (22) revealed by culture were AFB negative by direct smear microscopy. Among 47 culture positive while smear negative, 17 (36%) and 22 (48.6%) were from peripheral LED-FM and ZN using health facilities laboratories respectively (Table 5).

**Table 5:** Direct smear microscopy versus mycobacterial culture

| Microscopy                          |                     | Mycobacterial culture |              |     |              |
|-------------------------------------|---------------------|-----------------------|--------------|-----|--------------|
|                                     |                     | MTB positive          | MTB negative | NTM | Contaminated |
| AFB positive                        | HIV positive        | 11                    | 1            |     |              |
|                                     | HIV negative        | 39                    |              |     |              |
| AFB negative                        | HIV positive        | 17                    | 136          | 7   | 8            |
|                                     | HIV negative        | 30                    | 369          | 15  | 15           |
| <b>Health facility laboratories</b> |                     |                       |              |     |              |
| AFB positive                        | ZN intermediate     | 7                     |              |     |              |
|                                     | ZN peripheral       | 27                    |              |     |              |
|                                     | LED-FM intermediate | 6                     |              |     |              |
|                                     | LED-FM peripheral   | 10                    |              |     |              |
| AFB negative                        | ZN intermediate     | 5                     | 71           |     | 3            |
|                                     | ZN peripheral       | 22                    | 150          | 10  | 9            |
|                                     | LED-FM intermediate | 3                     | 119          | 4   | 3            |
|                                     | LED-FM peripheral   | 17                    | 165          | 8   | 8            |

Based on results of direct smear microscopy, reexamination of the smear and smear prepared after specimens processing and mycobacterial culture results (Table 3), the overall prevalence of smear negative pulmonary tuberculosis was 39.2%, 95% C.I [35.4%; 42.9%] and 46.4%, 95% C.I [39%; 53.7%] in HIV-positive TB suspects.

Based on the results of direct smear microscopy versus mycobacterial culture (Table 5), the sensitivity, specificity, negative and positive predictive value were calculated in the Table 6.

**Table 6:** Sensitivity, Specificity, PPV and NPV of smear microscopy

|                          | <b>Sensitivity (%)</b><br>[95% C.I] | <b>Specificity (%)</b><br>[95% C.I] | <b>PPV (%)</b><br>[95% C.I]  | <b>NPV (%)</b><br>[95% C.I]  |
|--------------------------|-------------------------------------|-------------------------------------|------------------------------|------------------------------|
| <b>Overall</b>           | <b>51.5</b><br>[47 ; 55]            | <b>99.8</b><br>[99.4 ; 100]         | <b>98</b><br>[96.9 ; 99.1]   | <b>91.4</b><br>[88.7 ; 94.1] |
| <b>Intermediate labs</b> | <b>61.9</b><br>[55.4 ; 68.4]        | <b>100</b>                          | <b>100</b>                   | <b>95.9</b><br>[93.2 ; 98.6] |
| <b>Peripheral labs</b>   | <b>56.5</b><br>[53.6; 59.4]         | <b>99.6</b><br>[99.1 ; 100]         | <b>97.2</b><br>[95.6 ; 98.8] | <b>88.9</b><br>[85.9 ; 91.9] |
| <b>LED-FM using labs</b> | <b>44.4</b><br>[39.1 ; 49.7]        | <b>99.6</b><br>[98.9 ; 100]         | <b>94.1</b><br>[91.6 ; 96.5] | <b>93.4</b><br>[90.8 ; 96]   |
| <b>ZN-using labs</b>     | <b>55.7</b><br>[50.1 ; 61.3]        | <b>100</b>                          | <b>100</b>                   | <b>81.7</b><br>[77.3 ; 86.1] |
| <b>HIV positive</b>      | <b>39.2</b><br>[32 ; 46]            | <b>100</b>                          | <b>100</b>                   | <b>89.8</b><br>[85.3 ; 94.3] |

#### 4.4. Performance of Xpert MTB/RIF test

Of the 97 culture positive, Xpert MTB/RIF revealed an overall of 77 (79%) whereas 3 cases detected by the Xpert MTB/RIF test were culture negative. Of the 28 HIV positive and culture positive, 20 (71%) tested MTB positive by Xpert MTB/RIF and the Xpert MTB/RIF was negative for all NTM growth in culture (Table 7).

**Table 7:** Xpert MTB/RIF versus culture by HIV sero-status

| Xpert MTB/RIF test |              | Mycobacterial culture |     |          |              |
|--------------------|--------------|-----------------------|-----|----------|--------------|
|                    |              | MTB positive          | NTM | Negative | Contaminated |
| HIV-Positive       | MTB positive | 20                    |     | 3        |              |
|                    | MTB negative | 8                     | 7   | 131      | 8            |
|                    | Error        |                       |     | 2        |              |
| HIV-Negative       | MTB positive | 57                    |     | 2        |              |
|                    | MTB negative | 11                    | 15  | 368      | 15           |
|                    | Error        | 1                     |     |          |              |

Based on the mycobacterial culture results, the overall increment yield of Xpert MTB/RIF test over the direct microscopy was 41.8%, 95% C.I [38; 45.5] and 47.6%, 95% C.I [40.2; 55] in HIV positive suspects. The sensitivity, specificity, positive and negative predictive values for Xpert MTB/RIF test were calculated in the Table 8.

**Table 8:** Sensitivity, Specificity, PPV and NPV of Xpert MTB/RIF test

|                       | <b>Sensitivity (%)</b><br><b>[95% C.I]</b> | <b>Specificity (%)</b><br><b>[95% C.I]</b> | <b>PPV (%)</b><br><b>[95% C.I]</b> | <b>NPV (%)</b><br><b>[95% C.I]</b> |
|-----------------------|--|--|------------------------------------|------------------------------------|
| <b>Overall</b>        | <b>80.2</b><br>[77.2 ; 83.2]               | <b>99</b><br>[98.2 ; 100]                  | <b>96.6</b><br>[95.2 ; 98]         | <b>97</b><br>[95.6 ; 98.4]         |
| <b>Smear positive</b> | <b>91.8</b><br>[84 ; 99.1]                 |  |                                    |                                    |
| <b>Smear negative</b> | <b>60</b><br>[56 ; 64]                     |  |                                    |                                    |
| <b>HIV positive</b>   | <b>71.4</b><br>[64.7 ; 78.1]               | <b>97.7</b><br>[95.5 ; 99.9]               | <b>86.9</b><br>[81.9 ; 91.8]       | <b>94.2</b><br>[90.7 ; 97.7]       |
| <b>HIV negative</b>   | <b>83.8</b><br>[80.4 ; 87.1]               | <b>99.4</b><br>[98.7 ; 100]                | 96.6<br>[94.9 ; 98.2]              | <b>97</b><br>[95.4 ; 98.5]         |



## 5.0.DISCUSSION

Tuberculosis remains a global public health emergency which disproportionately affects the poorer countries of the world. In these countries with high tuberculosis burden, the laboratory infrastructure for the diagnosis of infectious diseases is not adequately resourced. The most frequently available diagnostic technique for TB to peripheral and intermediate levels of health services is sputum smear microscopy. Though inexpensive, highly specific, rapid and relatively simple, sputum smear microscopy is associated with low and variable sensitivity and is exacerbated in HIV- TB coinfecting patients. Consequently, TB case detection rates are still below the 2005 DOT strategy target in most high-burden and low-income countries [100].

In Rwanda, conventional sputum smear microscopy using ZN staining is mostly used and the replacement of this technique by LED-FM which uses auramine-O stain is being implemented. Currently, LED-FM technique has been implemented in 30% of TB diagnostic health facilities. The LED-FM technique is thought to be more sensitive, rapid and relatively simpler than ZN technique and this could result in increasing the TB case detection rate. In addition, NTP has introduced Xpert MTB/RIF test, a new molecular tool, in 16 health facilities in order to increase TB detection rate especially in HIV pulmonary TB suspects. The present study aimed to determine the accuracy of routine sputum smear microscopy in detection of pulmonary TB in peripheral and intermediate health facility laboratories and to assess incremental yield of Xpert MTB/RIF test.

The study found, overall sensitivity of sputum smear microscopy to be 51.5 %, 95% C.I [47%; 55%]; specificity of 99.8%, 95% C.I [99.4%; 100%]; PPV of 98%, 95% C.I [96.9%; 99.1%] and NPV of 91.4%, 95% C.I [88.7%; 94.1%]. This sensitivity was slightly low compared to 70% which is achieved if sputum smear microscopy is optimized [5]. The sensitivity found in this study was in the range of findings from several studies where the sensitivities of conventional

ZN microscopy ranged from 32 to 94% and that the sensitivities of fluorescence microscopy ranged from 52 to 97%, with the fluorescent method being on average 10% more sensitive than light microscopy [4]. However the findings of the current study showed the performance of LED-FM (sensitivity of 44.4%, 95% C.I [39.1; 49.7], specificity of 99.6%, 95% C.I [98.9%; 100%], PPV of 97.2%, 95% C.I [95.6%; 98.8%] and NPV of 93.4%, 95% C.I [90.8%; 96%]) to be poor compared to the conventional ZN techniques, (sensitivity of 55.7%, 95% C.I [50.1%; 61.3%], specificity of 100%, PPV of 100% and NPV of 81.7%, 95% C.I [77.3; 86.1]). These results differ from many studies where LED-FM increases an average of 10% of sensitivity over the conventional ZN technique [33, 35, 38-40, 108]. Other studies have shown the equal sensitivity or low specificity of LED-FM compared to conventional ZN technique [39, 43-45]. In these studies, readers had no previous experience with fluorescence microscopy, which is the most likely explanation for performance differences compared with other studies and this highlights the importance of adapting training intensity according to the level of operator proficiency. In Rwanda, peripheral or intermediate laboratories have only one to two laboratory technicians who received adequate training on LED-FM technique and they are not the only sputum smear readers. This could explain the poor performance of LED-FM compared to conventional ZN technique observed in our study. Indeed, in Rwanda, most of laboratory technicians from intermediate and peripheral laboratory were trained and experienced in conventional ZN technique. This obviates the need for extensive training when introducing LED-FM in a new setting. However the specificity and positive predictive value find in this study were high and consistent with those of several studies where they ranges from 99.5% to 100% [108-110].

The accuracy of sputum smear microscopy at intermediate health facility laboratories (sensitivity of 61.9%, 95% C.I [55.4%; 68.4%]; specificity of 100%; PPV of 100% and NPV of 95.9%, 95%

C.I [93.2%; 98.6%]) was slightly higher than peripheral laboratories (sensitivity of 56.5%, 95% C.I [53.6%; 59.4%]; specificity of 99.6%, 95% C.I [99.1%; 100%]; PPV of 97.2%, 95% C.I [95.6% ; 98.8%] and NPV of 88.9%, 95% C.I [85.9%; 91.9%]). This could be explained by the fact that intermediate laboratories have more experienced laboratory technicians and low workload compared to peripheral health facility laboratories. In the present study, 65.8 % (427) participants and 78.3% (76) pulmonary tuberculosis cases were from peripheral health facilities. The fact that the peripheral health facilities are closer to the community, explains the high workload within these facilities. Indeed most of the technicians at peripheral health facilities are mostly trained, mentored and supervised by intermediate laboratory technologists.

The sensitivity of sputum smear microscopy in HIV positive participants (39.2%, 95% C.I [32%; 46%]) was found to be low and consistent with several studies, where its ranges from 30 to 48% [111-113]. The poor performance of sputum smear microscopy in HIV patients is explained by the fact that pulmonary tuberculosis in these patients bears many similarities to childhood tuberculosis, both are paucibacillary, involve hilar and mediastinal lymph nodes and lack cavitations [113].

The overall prevalence of smear negative pulmonary tuberculosis was 39.2%, 95% C.I [35.4%; 42.9%] and 46.4%, 95% C.I [39%; 53.7%] in HIV-positive TB suspects, this was consistent with the findings in a review by Colebunders *et al.* [114], where each smear positive case was associated with 1.22 cases of smear negative in HIV negative and 43% in a study carried out in Lusaka, Zambia by Elliot *et al.*[115]. The level of immunosuppression among HIV-infected patients affect significantly the results of smear. Less severely immunocompromised HIV-positive patients tend to have classic cavitary tuberculosis which is smear-positive, as the level of immunocompromise increases with advancing HIV disease, atypical pulmonary features predominate and smear examinations prove less sensitive [114].

The overall sensitivity of Xpert MTB/RIF test for the detection of *Mycobacterium tuberculosis complex*, 80.2% [77.2%; 83.2%] was slightly low compared to the pooled median sensitivity of Xpert compared with culture in a Cochrane review where it was 88%, 95% CI [83%; 92%]. However the specificity of 99%, 95% C.I [98.2% ; 100%] was consistent with the pooled median specificity in Cochrane review 98%, 95% C.I [97%; 99%] and the sensitivity in smear-positive cases was in the same range 91.8% [84% ; 99.1%] versus 98% [97 to 99] and 60%, 95% C.I [56 ; 64] versus 68%, 95% C.I [60 ; 75] in smear-negative cases [9].

In HIV-positive TB cases, the sensitivity appeared lower than in HIV-negative patients 71.4%, 95% C.I [64.7 ; 78.1] versus 83.8%, 95% C.I [80.4 ; 87.1], whereas there was little difference in specificity. The findings were consistent with Cochrane review where in HIV positive the pooled median sensitivity was 76%, 95% C.I [63; 85] and 89%, 95% C.I [81; 94]) in HIV negative [9]. However it was slightly low compared to other recently published studies which reported a high Xpert sensitivity in HIV-positive patients [82-85]. Across all these studies, specificity was high and Xpert offered significant advantages over smear. In this study the overall increment yield of Xpert MTB test over the direct microscopy was 41.8%, 95% C.I [38; 45.5] and 47.6%, 95% C.I [40.2; 55] in HIV positive tuberculosis suspects. Indeed among seven rifampicin resistant identified by Xpert, five were smear negative.

## **6.0. CONCLUSION AND RECOMMENDATIONS**

### **6.1. Conclusion**

- The sensitivity of sputum smear microscopy in Rwandan peripheral and intermediate health facility laboratories is still low [39.1% to 68.4%] compared to [70%], the target set by WHO to be achieved by 2005.
- The accuracy of sputum smear microscopy in health facility laboratories using LED-FM microscopy is lower than in health facility laboratories using ZN technique in Rwanda, sensitivity of 44.4%, 95% C.I [39.1; 49.7] versus 55.7%, 95% C.I [50.1; 61.3] respectively.
- The use of sputum smear microscopy alone in peripheral and intermediate health laboratories for pulmonary tuberculosis detection could lead to underdiagnoses of more than 50% of tuberculosis cases (smear microscopy detected 51 over 102 tuberculosis cases identified by culture and/or Xpert test).
- The increment yield of Xpert MTB/RIF over the microscopy is significantly high in regards to gold standard, overall in tuberculosis suspects 41.8%, 95% C.I [38; 45.5] and 47.6%, 95% C.I [40.2; 55] in HIV positive tuberculosis suspects.

### **6.2. Recommendations**

#### **To the National Reference Laboratory:**

- In order to increase the sensitivity of sputum smear microscopy, especially in LED-FM using health facilities, intensive and systematic training is urgently required for all laboratory technicians who examine the sputum smear in these sites. The phase in of LED-FM should be highly monitored and before complete shifting from ZN, all laboratory technicians should be adequately trained.

- Given the poor stability of stains used in fluorescence microscopy (Auramine O and KMnO<sub>4</sub>), the NRL should ensure the quality of these reagents being used at sites by regular supervision.
- Strong external quality assurance system (slides rechecking, intensive supervision and proficiency panel testing) should be applied in all sites implementing the LED-FM
- Ensure the LED-FM are functioning properly and their maintenance are being done according to manufacturer's recommendation at health facilities using this technique.

**To the National Tuberculosis control Program:**

- In order to reduce workload and increase the sensitivity of smear microscopy at health facility level, there is a need to revise criteria for TB suspects' selection (to capture only the real pulmonary TB suspects). Even though we used more sensitive methods for TB diagnosis during this study, only 15% of TB suspects were confirmed to be pulmonary tuberculosis cases.
- Given the poor performance of sputum smear microscopy at health facility level, it's highly recommended to use Xpert MTB/ RIF test in all smears negative cases with prominent signs and symptoms of pulmonary tuberculosis (all real pulmonary TB suspects), in order to increase the case detection and break the transmission chain of TB.

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## **APPENDICES**

### **Appendix 1. Consent form for research participants**

**Title of the study:** The Accuracy of sputum smear microscopy in detection of pulmonary tuberculosis in peripheral and intermediate health facility laboratories in Rwanda and incremental yield of Xpert MTB RIF test

#### **Investigator**

Ngabonziza Semuto Jean Claude **Phone** : +250788740490/+254717134945

#### **Purpose**

You are invited to participate in this research study. The purpose of this study is to determine the accuracy of routine sputum smear microscopy in detection of pulmonary tuberculosis in peripheral and intermediate health facility laboratories and to assess the incremental yield of Xpert MTB/RIF test on routine microscopy results.

#### **Participants**

You are invited to participate in this study only if you are suspected to have pulmonary tuberculosis and you are sent for sputum smear microscopy by a medical officer.

#### **Procedures**

If you consent to participate, you will be requested to provide 5ml of sputum specimens to be used for diagnosis of pulmonary tuberculosis.

**Risks:** No any risk has been identified.

#### **Benefits**

You will get culture and Xpert MTB RIF test **and if the results from these test show evidence of tuberculosis disease, you will be treated free of charge.** The data from this study may help health facility, planner and other researchers to offer the best services in diagnosis and management of pulmonary tuberculosis in the future.

### **Confidentiality**

Your sputum specimen will be assigned a code number and the key to the code will be maintained by the principal investigator. Records of this study will be stored in a locked file cabinet at National Reference Laboratory and kept for a minimum of 5 years after the completion of the study. Your identity will not be revealed to any unauthorized persons and will be protected to the extent allowed by law. You will not be personally identified in any reports or publications that may result from this study.

### **Costs and Compensation**

Your participation in the study will take approximately 5 minutes. You will not be paid for the study procedure. You will not be compensated for the sputum specimen.

### **Right to refuse or withdraw**

If you choose to participate or withdraw from the study at any time you will still receive treatment at the health center.

If the study design or use of the data is to be changed, you will be so informed and your consent re-obtained. You will be told of any significant new findings developed during the course of this study, which may relate to your willingness to continue participation.

### **Questions**

If you have any question, please ask me. If you have any additional questions later, contact the researcher, Ngabonziza Semuto Jean Claude, National Reference Laboratory, RBC; Telephone: +250788740490/+254717134945; Email: [cldesemuto@gmail.com](mailto:cldesemuto@gmail.com). **If you have any question about your right as participants** please contact the chairperson of Rwanda Ethics and Research Committee, Dr. Jean Baptiste MAZARATI Telephone: (+250)788309807, P.O. Box 84 Kigali, Email: [rnc@moh.gov.rw](mailto:rnc@moh.gov.rw) and the KNH/UoN-ERC on telephone number (254-020) 2726300 Ext 44355, P.O. Box 19676 code 00202, Email: [uonknh\\_erc@uonbi.ac.ke](mailto:uonknh_erc@uonbi.ac.ke).

**My signature below indicates that I have decided to volunteer as a research subject and that I have read and I have received a copy of this consent form.**

Signature (or thumb print) of participant

..... Date .....

Witness

Name.....Date.....Signature.....

Signature of Investigator.....Date.....

## **Appendix 2. Consent form for research participants (Kinyarwanda version)**

UBUSHAKE BUSESUYE MU GUKORANA MU BUSHAKASHATSI

**Umutwe y’ubushakashatsi “Ubushobozi bwibizamini bikoreshwa mugusuzuma indwara y’igituntu cyo mubihaha kubitaro no kubigo nderabuzima mu Rwanda”**

### **Umushakashatsi**

Ngabonziza Semuto Jean Claude **Phone** : +250788740490/+254717134945

### **Intego z’ ubushakashatsi**

Mutumiwe kugira uruhare muri ubu bushakashatsi bufite intego yo kureba ubushobozi ibizamini bikoreshwa mugusuzuma indwara y’igituntu cyo mu bihaha ku Bitaro no Kubigo nderabuzima mu Rwanda. Ubu bushakashatsi buzasuzuma icyo ikizamini gishyasha cyitwa Xpert MTB RIF test cyakongerwa mu gusuzuma igituntu cyo mu bihaha kuri ibyo Bitaro n’ibigo nderabuzima byatoranijwe.

### **Abatumiwe kugira uruhare mu bushakashatsi**

Umuntu wese ufite ibimenyetso by’indwara y’igituntu cyo mu bihaha, akaba yasabiwe na muganga gutanga igikororwa muri laboratwari kugirango bamusuzume indwara igituntu.

### **Ibyo musabwa**

Niba mwemeye kugira uruhare muri ubu bushakashatsi murasabwa gutanga mililitiro 5 zigikororwa, zo gukoreshwa mugusuzuma indwara y’igituntu cyo mu bihaha.

**Ingaruka:** Ntandaruka nimwe ihari mugutanga igikororwa.

### **Inyungu**

Muzakorera ibizamini bwo guhinga agakoko gatera indwara y'igituntu cyo mu bihaha n'ikizamini cya Xpert MTB RIF test gisuzuma indwara y'igituntu kikareba uko yumva imiti ikivura kubuntu.

### **Ibanga**

Igikororwa utanze gihabwa umubare wihariye, nta mazina azakoreshwa. Umwirondoro kumubare w'ibanga ubikwa n'umushakashatsi wenyine. Inyandiko zizava muri ubu bushakashatsi zizabikwa ahantu hafunze muri laboratwari nkuru y'igihugu mugihe cy'imyaka itanu. Nta muntu numwe uzabona ibisuzo byawe atabihereye uburenganzira nkuko biteganwa n'amategeko agenga ubushakashatsi. Ntahantu hazagaragara umwirondoro wawe haba muri raporo cyangwa mugutangaza ibyavuye muri ubu bushakashatsi.

### **Ikiguzi cyo kugira uruhare mubushakashatsi**

Kugira uruhare muri ububushakashatsi biragufata iminota igera kuri itanu. Ntabwo uribwishyurwe kandi ntakiguzi cy'igikororwa gitangwa.

### **Uburenganzira bwo kwanga cyangwa kuva mubushakashatsi.**

Ufite uburenganzira bwo kwemera, kwanga cyangwa kuvamo igihe icyo aricyo cyose mubishakiye. Muravurwa neza nkuko biteganywa mwaba mwemeye, mwanze cyangwa se muvuye muri ubu bushakashatsi.

Hagize igihinduka muri gahunda y'ubu bushakashatsi, muzabimenyeshwa kandi hasabwe n'ubushake bwanyu. Muzamenyeshwa icyo ububushakatsi bwagezeho.

### **Ibibazo**

Niba hari ikibazo mufite mwabaza umushakashatsi. Mugize ibyo mwifuza kumenya nyuma mwabaza NGABONZIZA S. Jean Claude kuri tel: +250788740490/+254717134945; Email: [cldesemuto@gmail.com](mailto:cldesemuto@gmail.com). Muramutse mugize ikibazo kuburenganzira bwanyu, mwabaza umuyobozi uhagarariye ubushakatsi mu Rwanda “Rwanda National Ethics and Research Committee” Dr. MAZARATI J. Baptiste Telephone (+250) 788309807, P.O. Box 84 Kigali, Email: [nec@moh.gov.rw](mailto:nec@moh.gov.rw). Mushobora kubaza nabayobozi ba KNH/UoN-ERC on telephone number (254-020) 2726300 Ext 44355, P.O. Box 19676 code 00202, Email: [uonknh\\_erc@uonbi.ac.ke](mailto:uonknh_erc@uonbi.ac.ke).

**Umukono wanjye uvuga ko nasobanukiwe kandi nemeye kugira uruhare muri ubu bushakashatsi.**

Umukono .....italiki .....

**Umuhamya**

Amazina.....Umukono.....Italiki

Umushakashatsi.....italiki.....

### **Appendix 3. Assent form of participants below 21 years old**

**Title of the study:** The Accuracy of sputum smear microscopy in detection of pulmonary tuberculosis in peripheral and intermediate health facility laboratories in Rwanda and incremental yield of Xpert MTB RIF test

#### **Investigator**

Ngabonziza Semuto Jean Claude **Phone** : +250788740490/+254717134945

#### **Purpose**

You are invited to participate in this research study. The purpose of this study is to determine the accuracy of routine sputum smear microscopy in detection of pulmonary tuberculosis in peripheral and intermediate health facility laboratories and to assess the incremental yield of Xpert MTB/RIF test on routine microscopy results.

#### **Participants**

You are invited to participate in this study only if you are suspected to have pulmonary tuberculosis and you are sent for sputum smear microscopy by a medical officer.

#### **Procedures**

If you assent for him or her to participate, he or she will be requested to provide 5ml of sputum specimens to be used for diagnosis of pulmonary tuberculosis.

#### **Risks**

No any risk has been identified.

#### **Benefits**

He or she will get culture and Xpert MTB RIF test results free of charge and data from this study may help health facility, planner and other researchers to offer the best services in diagnosis and management of pulmonary tuberculosis in the future.

### **Confidentiality**

His or her sputum specimen will be assigned a code number and the key to the code will be maintained by the principal investigator. Records of this study will be stored in a locked file cabinet at National Reference Laboratory and kept for a minimum of 5 years after the completion of the study. His or her identity will not be revealed to any unauthorized persons and will be protected to the extent allowed by law. He or she will not be personally identified in any reports or publications that may result from this study.

### **Costs and Compensation**

His or her participation in the study will take approximately 5 minutes. He or she will not be paid for the study procedure. He or she will not be compensated for the sputum specimen.

### **Right to refuse or withdraw**

If you choose for him or her to participate or withdraw from the study at anytime you will still receive treatment at the health center.

If the study design or use of the data is to be changed, you will be so informed and your assent re-obtained.. You will be told of any significant new findings developed during the course of this study, which may relate to your willingness to continue participation.

### **Questions**



If you have any question, please ask me. If you have any additional questions later, contact the researcher, Ngabonziza Semuto Jean Claude, National Reference Laboratory, RBC; Telephone: +250788740490/+254717134945; Email: [cldesemuto@gmail.com](mailto:cldesemuto@gmail.com). **If you have any question about your right as participants** please contact the chairperson of Rwanda Ethics and Research Committee, Dr. Jean Baptiste MAZARATI Telephone: (+250)788309807, P.O. Box 84 Kigali, Email: [rnc@moh.gov.rw](mailto:rnc@moh.gov.rw) and the KNH/UoN-ERC on telephone number (254-020) 2726300 Ext 44355, P.O. Box 19676 code 00202, Email: [uonknh\\_erc@uonbi.ac.ke](mailto:uonknh_erc@uonbi.ac.ke).

**My signature below indicates that I have decided to volunteer as a research participants and that I have read and I have received a copy of this assent form.**

Signature (or thumb print) of participants' parent

..... Date .....

Name.....Date.....Signature.....

Signature of Investigator.....Date.....

#### **Appendix 4. Assent form of participants bellow 21 years old (Kinyarwanda version)**

UBUSHAKE BUSESUYE MU GUKORANA MU BUSHAKASHATSI

**Umutwe y’ubushakashatsi “Ubushobozi bwibizamini bikoreshwa mugusuzuma indwara y’igituntu cyo mubihaha kubitaro no kubigo nderabuzima mu Rwanda”**

#### **Umushakashatsi**

Ngabonziza Semuto Jean Claude **Phone** : +250788740490/+254717134945

#### **Intego z’ ubushakashatsi**

Mutumiwe kugira uruhare muri ubu bushakashatsi bufite intego yo kureba ubushobozi ibizamini bikoreshwa mugusuzuma indwara y’igituntu cyo mu bihaha ku Bitaro no Kubigo nderabuzima mu Rwanda. Ubu bushakashatsi buzasuzuma nicyo ikizamini gishyasha cyitwa Xpert MTB RIF test cyakongerwa mu gusuzuma igituntu cyo mu bihaha kuri ibyo Bitaro n’ibigo nderabuzima byatoranijwe.

#### **Abatumiwe kugira uruhare mu bushakashatsi**

Umuntu wese ufite ibimenyetso by’indwara y’igituntu cyo mu bihaha, akaba yasabiwe na muganga gutanga igikororwa muri laboratwari kugirango bamusuzume indwara igituntu.

#### **Ibyo musabwa**

Niba mwemeye ko umwana wanyu yagira uruhare muri ubu bushakashatsi yasabwa gutanga militiro 5 zigikororwa, zo gukoreshwa mugusuzuma indwara y’igituntu cyo mu bihaha.

**Ingaruka:** Ntanganuka nimwe ihari mugutanga igikororwa.

#### **Inyungu**

Azakorerwa ibizamini bwo guhinga agakoko gatera indwara y'igituntu cyo mu bihaha n'ikizamini cya Xpert MTB RIF test gisuzuma indwara y'igituntu kikareba uko yumva imiti ikivura kubuntu.

### **Ibanga**

Igikororwa utanze gihabwa umubare wihariye, nta mazina azakoreshwa. Umwirondoro kumubare w'ibanga ubikwa n'umushakashatsi wenyine. Inyandiko zizava muri ubu bushakashatsi zizabikwa ahantu hafunze muri laboratwari nkuru y'igihugu mugihe cy'imyaka itanu. Nta muntu numwe uzabona ibisuzo byiwe atabihereye uburenganzira nkuko biteganwa n'amategeko agenga ubushakashatsi. Ntahantu hazagaragara umwirondoro we haba muri raporo cyangwa muguntangaza ibyavuye muri ubu bushakashatsi.

### **Ikiguzi cyo kugira uruhare mubushakashatsi**

Kugira uruhare muri ububushakashatsi biragufata iminota igera kuri itanu. Ntabwo uribwishyurwe kandi ntakiguzi cy'igikororwa gitangwa.

### **Uburenganzira bwo kwanga cyangwa kuva mubushakashatsi.**

Ufite uburenganzira bwo kwemera, kwanga cyangwa kuvamo igihe icyo aricyo cyose mubishakiye. Aravurwa neza nkuko biteganywa mwaba mwemeye, mwanze cyangwa se muvuye muri ubu bushakashatsi.

Hagize igihinduka muri gahunda y'ubu bushakashatsi, muzabimenyeshwa kandi hasabwe n'ubushake bwanyu. Muzamenyeshwa icyo ububushakatsi bwagezeho.

### **Ibibazo**

Niba hari ikibazo mufite mwabaza umushakashatsi. Mugize ibyo mwifuza kumenya nyuma mwabaza NGABONZIZA S. Jean Claude kuri tel: +250788740490/+254717134945; Email: [cldesemuto@gmail.com](mailto:cldesemuto@gmail.com). Muramutse mugize ikibazo kuburenganzira bwanyu mwabaza umuyobozi uhagarariye ubushakatsi mu Rwanda “Rwanda National Ethics and Research Committee” Dr. MAZARATI J. Baptiste Telephone (+250) 788309807, P.O. Box 84 Kigali, Email: [rnc@moh.gov.rw](mailto:rnc@moh.gov.rw). Mushobora kubaza nabayobozi ba KNH/UoN-ERC on telephone number (254-020) 2726300 Ext 44355, P.O. Box 19676 code 00202, Email: [uonknh\\_erc@uonbi.ac.ke](mailto:uonknh_erc@uonbi.ac.ke).

**Umukono wanjye uvuga ko nasobanukiwe kandi nemeye ko nemereye umwana kugira uruhare muri ubu bushakashatsi.**

Amazina y’umwana ahagarariye.....

**Umubyeyi uhagarariye umuntu uri muni y’ imyaka 21**

**Amazina.....Umukono.....Italiki.....**

Umushakashatsi.....italiki.....

**Appendix 5. Data collection tool**

Patient identification number  Age  Sex: Male  Female

Health facility.....

Duration of illness (days)

**Signs and symptoms**

Fever  Chills  Chest pain  Dyspnea  Fatigue  Malaise

Weight loss  Night sweats  Productive cough  haemoptysis

Lack of appetite

**Chest X-ray results suggestive of pulmonary tuberculosis**

Positive  Negative  Unknown  Not done

**HIV status**

Negative  Positive  Unknown

**Appearance & quantity of sputum specimen**

Purulent  Mucoid  Mucopurulent  Bloody stained

Salivary  Volume of sputum  ml

Size of direct smear (L×W)  ×  mm

**Staining method:** ZN      Auramine

**Examination time (min):**

**Result:** Negative       Positive       If positive Grade

**Re-examination of the direct smear**

**Quality of staining:** Good       Poor

**Result:** Negative       Positive       If positive Grade

**Examination of concentrated smear**

**Result:** Negative       Positive

**Xpert MTB RIF test**

**Results:** Negative       Positive       Invalid       If positive RIF

**Culture results:**

**LJ:**

Days to result

**Result:** Negative       Positive       Contaminated

If positive number colonies

**Liquid culture (MGIT):**

Days to result

**Result:** Negative  Positive  Contaminated

**Identification if LJ and/or MGIT positive**

**ZN on colonies and/or MGIT:** AFB Negative  AFB Positive

**Results:** MTB  MOTT

## Appendix 6. Xpert MTB/RIF MTB/RIF test procedure

- i. Label each XpertMTB/RIF cartridge with sample I.D. (Write on the side of the cartridge or affix label). Do not put label on the lid of the cartridge or obstruct the cartridge barcode.
- ii. Add 1.5ml of XpertMTB/RIF sample reagent to 0.5ml sample deposit.
- iii. Shake vigorously 20 times then stand for 10 minutes. One back and forth movement is a single shakes. After 10 min, shake 20 times then stand for 5 min.
- iv. Use the sterile pipette provided in the cartridge kit to aspirate the liquefied sample until the liquefied sample meniscus, is above the minimum mark.
- v. Ensure that the liquefied sample being transferred to the cartridge has no bubbles as this may cause an error.
- vi. On the windows desktop, double click on the Xpert MTB/RIF® Dx shortcut, log on to the system software using your user name and password.
- vii. In the Xpert MTB/RIF® Dx software toolbar, click on the create test icon. Depending on your lab protocols scan the patient barcode then the cartridge barcode and the system fills the other boxes automatically i.e. select assay, reagent lot I.D, cartridge SN and Expiration date.
- viii. If your lab does not identify patients' using barcodes, then proceed to enter their patient I.D. manually.
- ix. Click on start test box. Type your username and password in the dialog box that appears.
- x. Open the instrument module door with the blinking green light and load the cartridge. Close the door. The module door latches shut. Once the test starts, the green light stops blinking. When the test finishes the light goes off.
- xi. Wait until the system releases the door lock then, open the module door and remove the



cartridge.

- xii. The results of Xpert MTB/RIF test are displayed on the computer screen and interpretation is done as shown in the table below

| <b>Xpert® MTB/RIF Readout</b>              | <b>Interpretation</b>   | <b>Report* (Suggested Minimal Language)</b>   |
|--|---|---|
| MTB DETECTED; RIF Resistance DETECTED      | MTB target is detected within sample.<br><br>A mutation in the <i>rpoB</i> gene has been detected. A full first and second line drug panel should be conducted. | MTBC detected.<br><br><i>rpoB</i> mutation detected; likely rifampin resistance; Confirmatory testing in progress OR isolate has been forwarded to a reference laboratory for confirmatory testing. |
| MTB DETECTED; RIF Resistance NOT DETECTED  | MTB target is detected within sample.<br><br>A mutation in the <i>rpoB</i> gene has not been detected.  | MTBC detected.<br><br>No <i>rpoB</i> mutation detected; likely rifampin susceptible.  |
| MTB DETECTED; RIF Resistance INDETERMINATE | MTB target is detected within sample.<br><br>A mutation in the <i>rpoB</i> gene could not be determined due to insufficient signal detection.                   | MTBC detected.<br><br>Insufficient MTB in the sample to allow determination of <i>rpoB</i> mutation result.   |
| MTB Not Detected                           | MTB target is not detected within the sample.   | MTBC not detected.  |

**\*RIF Results should be reported prior to culture confirmation.**

- xiii. Dispose of the cartridges in the appropriate specimen waste containers according to your institution's standard procedure.

## **Appendix 7. Digestion and decontamination of specimens by NaLC-NaOH**

- i. Transfer approximately 10 ml of the sputum into a pre-labeled (with appropriate Lab ID) 50 ml capacity sterile disposable plastic centrifuge tube. (If volume of sample is more than 10mL, it should be centrifuged and make to 10mL. If it is less than 5 mL, make the volume up to 10mL using sterile saline or distilled water).
- ii. Add an equal volume of NALC- NaOH/Sodium citrate solution.
- iii. Switch on the timer set for 15 minutes as soon as the first sample comes in contact with NALC-NaOH/citrate solution and another Timer on 15 minutes when last sample has been added.
- iv. Tighten the screw cap and mix the contents by a vortex mixer for 10- 15 seconds (Caution- violent vortexing is not recommended because denaturing of the NALC may take place).
- v. Allow the mixture to stand for 15 min to effect decontamination. Vortex lightly or hand mix/invert every 5-10 minutes.
- vi. Make sure the specimen is liquefied. If still mucoid, add small quantity of NALC powder (30-35 mg) directly into the tube. Mix well by vortex lightly for 5 sec.
- vii. After 15 minutes are over, dilute the digested-decontaminated specimen to the 45 ml mark with sterile phosphate buffer, pH 6.8. (This dilution helps to minimize the continuing action of Sodium hydroxide and to lower the specific gravity before centrifugation).
- viii. Tighten the centrifuge tube caps and mix well by swirling and inversion.
- ix. Centrifuge the tube at 3000 x g for 15 min using aerosol-free sealed centrifuge buckets.

- x. After centrifugation, carefully decant the supernatant into a splash proof discard flask containing a mycobactericidal disinfectant 5% Dettol or equivalent. Swab lip of the centrifuge tube with disinfectant soaked gauze/tissue and recap.
- xi. Resuspend sediment in 1 to 2 ml of sterile phosphate buffer, pH 6.8.
- xii. The resuspend sediment is now ready for making smears and for inoculation into MGIT, LJ slants and Blood agar for QC.

## Appendix 8. Ziehl Nelsen method

### Procedure

- i. Fix the smear of the specimen over the glass slide, either by heating or alcohol fixation.
- ii. Pour carbol fuchsin over smear and heat gently until fumes appear. Do not overheat and allow it to stand for 5 minutes, and then wash it off with water.
- iii. Pour 20% sulphuric acid, wait for one minute and keep on repeating this step until the slide appears light pink in color. Wash off with water.
- iv. Pour methylene blue, wait for two minutes, and again wash with water
- v. Allow it to air dry and examine under oil immersion lens.

### Result

Acid fast bacilli stain pink, straight or slightly curved rods, at times having beaded appearance. The background appears blue due to methylene blue.

### IUATLD-recommended grading of sputum smear microscopy results

| AFB counts                                  | Recording/reporting |
|---|---------------------|
| No AFB in at least 100 fields               | 0/Negative          |
| 1 to 9 AFB in 100 fields                    | Actual AFB counts   |
| 10 to 99 AFB in 100 fields                  | +                   |
| 1 to 10 AFB per field in at least 50 fields | ++                  |
| >10 AFB per field in at least 20 fields     | +++                 |

## Appendix 9. Auramine O staining and LED-FM method

### Procedure

- i. Fix the smear of the specimen over the glass slide, either by heating or alcohol fixation.
- ii. Flood the slide with auramine O (fluorochrome) stain, stain for 15 min.
- iii. Rinse the slide with water; drain excess water from the slide.
- iv. Flood with 0.5% acid-alcohol; decolorize for 30-60 s. (some protocols call for 2 min.)
- v. Rinse the slide with water; drain excess water from the slide.
- vi. Flood the slide with potassium permanganate or acridine orange; counter stain for 2 min; do not allow the slide to dry.
- vii. NOTE: Timing is critical during the counterstaining step with potassium permanganate.
- viii. Counterstaining for a longer time may quench the fluorescence of acid-fast organisms.
- ix. Rinse the slide with water; drain excess water from the slide.
- x. Air dry; do not blot.
- xi. Examine the smear with a fluorescent microscope.
- xii. Examine smears using the high power objective (40X, total magnification, X400); verify using the oil immersion objective (100X, total magnification, X 1,000). Some recommend screening with a 25X objective.

### Results

Mycobacteria are approximately 1 to 10  $\mu\text{m}$  long and typically appear as green fluorescence slender rods. However, they may also appear curved or bent, cocco-bacillary, or even filamentous.

The results from this staining procedure should be reported only if the positive control smears are acceptable.

**IUATLD-recommended grading of sputum smears LED-FM results**

| <b>Magnification</b> |             |              | <b>Report</b>                                |
|----------------------|-------------|--------------|--|
| <b>250×</b>          | <b>450×</b> | <b>1000×</b> |  |
| 0                    | 0           | 0            | No AFB seen                                  |
| 1-2/30F              | 2/70F       | 2/300F       | Doubtful, Repeat and report the exact number |
| 1-9/10F              | 2-18/50F    | 1-9/100F     | 1+   |
| 1-9/F                | 4-36/10F    | 1-9/10F      | 2+   |
| 10-90/F              | 4-36/F      | 1-9/F        | 3+   |
| >90/F                | >36/F       | >9/F         | 4+   |

## Appendix 10. SD TB Ag MTPT64 Procedure

### Specimen preparation

- *Liquid cultures*

100µl of sample taken from liquid cultures can be applied directly to the sample well (S) without use of the sample preparation procedure

- *Solid cultures*

- 3-4 colonies should be suspended in 100µl of extraction buffer prior to test.
- If there is condensation fluid of slant agar tubes, 100µl could be taken directly to the sample well (S)

### Procedure of the test

- Remove the test device from the foil pouch and place it on a flat, dry surface.
- Add 100µl of liquid culture or a suspended solid cultures in the buffer into the sample well (S).
- As the test begins to work, you will see purple color move across the result window in the test device
- Interpret the test results in 15 minutes after sample application.

### Interpretation of the test

- A color band will appear at left section of the result window to show that the test is working properly. This band is the Control Band
- The right section of the result window indicates the test results. If another color band appears at the right section of the result window, this band is the Test Band.

**Negative Result:** The presence of only control band (“C” band) within the result window indicates a negative result

**Positive Result:** The presence of two color band (“**T**” band and “**C**” band) within the result window, no matter which band appears first, indicates a positive result.

Note:

1. Depending on the MPT64 antigens concentration, the intensity on test line may vary.
2. A positive result will not change once it has been established at 15 minutes.

**Invalid Result:** if the control band is not visible within the result window after performing the test, the result is considered invalid. The direction may not have been followed correctly or the test may have deteriorated. It is recommended that the specimen should be re-tested.



**Appendix 11. KNH-UoN – ERC approval**

**Appendix 12. Rwandan –ERC approval**