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DIAGNOSTIC ACCURACY OF *Mycobacterium.tuberculosis* DNA AS DETECTED BY THE TOP ASSAY AMONG INDIVIDUALS WITH PAUCIBILLARY TB

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A RESEARCH DISSERTATION SUBMITTED TO THE DEPARTMENT OF IMMUNOLOGY AND MOLECULAR BIOLOGY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF A MASTER'S DEGREE IN IMMUNOLOGY AND CLINICAL MICROBIOLOGY OF MAKERERE UNIVERSITY

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DECLARATION

I Kenneth Mubiru declare that this dissertation is my original work and has never been submitted to any institution for any academic award**.**

Signature: ……Date: ...07/11/2023.....

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DEDICATION

I dedicate this dissertation to my family, friends, course mates and everyone who supported me academically and socially.

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TABLE CONTENTS

LIST OF ABBREVIATION

WHO	World Health Organization
MTB	*Mycobacterium tuberculosis*
MDR-TB	Multidrug-resistant tuberculosis
XDR-TB	Extensive-drug resistant tuberculosis
RIF	Rifampicin
INH	Isoniazid
DST	Drug Susceptibility Testing
PZA	Pyrazinamide
TURN-TB	Trace Ultra Result iNsight in TB screening
TOP	Totally Optimized PCR
BP	Base pairs
rpoB	beta subunit of RNA polymerase

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DEFINITION OF TERMS

rpoB gene	Codes for the RNA polymerase β subunit, which is the target of rifampicin, an essential drug in the treatment of tuberculosis and other mycobacterial infections
Trace result	The "trace" means that only the IS1081 and IS6110 targets were detected, but not the TB-specific regions in the rpoB gene.
Paucibacillary TB	Small amounts of Mycobacterium *tuberculosis* DNA which is detected by GeneXpert Ultra as MTB detected Low, Very low and "Trace"

ABSTRACT

Background: One of the major reasons leading to the increased incidence of TB is the lack of a diagnostic with high sensitivity that can be relied on to capture even patients with low burden TB. This had been attempted by increasing the sensitivity of Xpert MTB/RIF assay with the Xpert MTB/RIF Ultra cartridge; however it has led to PCR results that are not diagnostic(Trace-positive) that require further study so that the result could be interpreted correctly, provide the right treatment for patients, and prevent the progression to active stages of TB infection(1). 'Totally Optimized PCR (TOP)', is a highly sensitive new nucleic acid amplification test that utilizes a combination of efficient sample processing, novel target selection, modern primer design techniques, and an extended PCR for selective target isolation and amplification. The assay targets a gene (ponA1) involved in the assembly of peptidoglycans in the MTB bacterial wall which is highly specific to Mycobacteria in the MTB complex and therefore it is not affected by background noise (2).

Methods: A nested cross-sectional study in Trace Ultra Result iNsight in TB screening (TURN-TB) study was carried out to evaluate the diagnostic accuracy of *Mycobacterium tuberculosis* DNA as detected by the top assay among individuals with paucibillary TB.

A total of 71 samples were tested on TOP assay, GeneXpert ultra tests were already done on the same specimens, 66 samples had valid TOP assay results and 5 had indeterminate results 30/66 had both sputum GeneXpert Ultra and sputum culture done.

Results: Relative to sputum Xpert Ultra, the sensitivity of TOP assay was 73.53% (n/N=25/34, 95% CI, 62.89-84.17) and specificity was 84.38% (n/N=27/32, 95% CI, 75.62-93.13), 45.46% (30/66) samples tested with TOP had both sputum Xpert Ultra and culture results which were compared to the microbiological reference standard (incorporating both sputum Xpert and culture), among these patients, 23.33% (7/30) had both sputum Xpert Ultra and culture positive results, among these cases, 71.43% (5/7) were detected by TOP assay, while 28.57% were not detected. Furthermore, 40% (12/30) of the patients had both sputum Xpert Ultra and culture negative results. Among these negative cases, 66.67% (8/12) were also negative on TOP, however, 33.33% (4/12) were considered false positive compared to the microbiological reference standard. The diagnostic sensitivity of TOP assay consistently increased in patients with higher sputum mycobacterial loads, as indicated by the semi-quantitative results of sputum Xpert ultra. Specifically, among the 22 cases with Xpert Ultar Low results, 90.91% (20/22) were detected by TOP, for the 11 cases with Very Low results, 45.45% (5/11) were detected by the TOP assay,. Finally, one case with a Trace-positive result was not detected by TOP.

Conclusion Overall; these results demonstrate that the TOP assay shows moderate sensitivity and specificity when compared to sputum Xpert Ultra, additionally, the assay's sensitivity is influenced by the sputum mycobacterial load, with higher loads correlating to increased detection rates,

CHAPTER ONE: INTRODUCTION

1.1Background

Tuberculosis (TB) one of the most lethal diseases in human history was first elucidated by Robert Koch (1843–1910) in 1882 (3). Koch developed a specific staining method based on methylene blue combined with red/brown counterstaining of host tissues with *Mycobacterium tuberculosis* (*Mtb*) which allowed visualization of bacteria not only in cultures, but also in tissues(4). Tuberculosis (TB) is an infectious respiratory disease that seriously endangers health. Despite the availability of a vaccine for nearly 100 years, the global infection rate of *Mycobacterium.tuberculosis* (MTB) is still approximately one in three people (5). The pathogen is eliminated in only 10% of people, but after it has successfully evaded the host immune response, it progresses to infection or remains dormant in old lesions making the infection hard to control(6), (7). It has been decades without the introduction of new antibiotics to treat Tuberculosis, and several antibiotic candidates are currently undergoing clinical investigation. TB treatment requires prolonged combination chemotherapy with several drugs. Moreover, monitoring the success of therapy is questionable due to the lack of reliable biomarkers(8).

Delayed diagnosis is a crucial problem in global TB control programs (WHO 2017). Bacteriological methods currently used to diagnose TB in endemic countries take up to 6 and 8 weeks, which poses a threat to the populations living with TB patients in increasing the rate of transmission. The presence of a heterogeneous population of *Mycobacterium tuberculosis*, the causative agent of TB, is among the reasons for delayed diagnosis by bacteriological methods since most of the available and cost effective techniques target common features that are possessed by most of the species(9). Previously, using a diagnostic luciferase reporter phage assay that can detect non-replicating bacilli in patient sputum,30 additional positive samples were identified, which failed to grow on standard Lowenstein–Jensen (LJ) agar medium(10).Currently available methods for measuring bacterial load have significant drawbacks. Sputum smears are rapid and cheap but have a sensitivity limit of 10,000 bacteria per ml sputum (11) and cannot differentiate between live and dead bacilli. Liquid culture methods for sputum bacterial load enumeration require decontamination which may not be effective when the patient is on anti-TB drugs; this reduces on the number of viable bacilli(12). The time to positivity of liquid cultures is dependent on the bacterial burden of the sputum inoculum. Quantitative culturing does not require decontamination but requires

extensive laboratory operator time, the *Mycobacterium tuberculosis* colonies grow slowly, and there is difficulty in obtaining single colonies due to bacterial cording. All culture-based methods are affected by contamination with other microorganisms present in the sample and viable non-cultivatable *M. tuberculosis* organisms.

Newly developed molecular assays significantly shorten the "sample-to-treatment" cascade of care and approach the sensitivity of culture. However, these tests are still best suited for detection of TB at all stages impacting on the treatment outcome. The available tests can target both DNA and RNA of *Mycobacterium tuberculosis* such as MBLA, MTB/RIF ultra, Line probe assay and Totally Optimized PCR (TOP) TB assay(13, 14). The Xpert MTB/RIF Ultra (Xpert Ultra) assay was developed to overcome the limited sensitivity of Xpert in the detection of pulmonary tuberculosis and limited accuracy of rifampicin resistance detection by incorporating two different multicopy amplification targets (IS6110 and IS1081)(15) which uses improved assay chemistry and cartridge design(16). However Xpert MTB/RIF Ultra produces trace results (Trace-positive) which are difficult to interpret hence affecting treatment outcome of patients with that result. TOP TB assay has been reported to produce a sensitivity close to 100% compared to culture in a study done in Mbarara, Uganda however the specificity was 35% (17) compared to culture but it detected many more positive samples than culture that were confirmed-positive by a second PCR followed by DNA sequencing, suggesting that TOP TB may be more sensitive than culture and other PCRs that approach the sensitivity of culture. TOP assay targets gene (ponA1) involved in the assembly of peptidoglycan in the *M.tuberculosis* bacterial wall (18, 19). The assay's diagnostic primer set (3-ponA-F/R) targets sequences unique to all species in the *M. tuberculosis* complex (19).If the technical performance of TOP is verified in a longitudinal study, then it can be used as a TB rule out test.

1.2 Problem statement

There is limited data about the technical performance of totally optimized PCR (TOP) assay in detecting MTB DNA in individuals with paucibacillary TB.

A pilot study done in Brazil in 2014 and a cross sectional study done in Boston in the same year assessed the analytical specificity against non-tuberculous mycobacteria (NTM) and estimated the diagnostic accuracy of TOP which they compared the TOP results to culture and a composite reference standard (CRS) (20). They found out that TOP exhibited no cross reactivity against NTM. The pilot demonstrated feasibility and 100% (95% CI 85–100) sensitivity in predominantly smear-positive specimens; TOP's specificity against solid media culture was low (58%, 37–77) but improved against a CRS (93%, 68–100). Similarly, when using the CRS in the Boston study, TOP (88%, 1–99) had greater sensitivity than solid or liquid media culture (25%, 3–65) and similar specificity (both 100%,93–100)(20), however their limitation was that they compared the TOP results against culture which is the gold standard in TB diagnosis, as four of the specimens tested were culture-positive/TOP-negative which could not be well explained whether it was the PCR inhibition that could have led to the false negative result.

A proof of concept study was done in 2016 in Mbarara, Uganda to detect and quantify *Mycobacterium tuberculosis* in the sputum of culture negative HIV infected pulmonary Tuberculosis suspects using TOP assay(17), they found out that using culture as reference, TOP had 100% sensitivity but 35% specificity. Against a composite reference standard, the sensitivity of culture (27%) and Xpert MTB/RIF (27%) was lower than TOP (99%), with similar specificity (100%, 98% and 87%, respectively) which showed that TOP assay was accurately detecting M.*tuberculosis* however their limitation was that they used low-volume, discarded and stored specimens from the existing diagnostic clinical study which could have compromised on the technical performance of the TOP assay(17).

The few studies done by Guillermo Madico and Jones Lopez were focused on culture negative specimens however the proposed study seeks to evaluate the presence and persistence of MTB DNA targets in the sputum of individuals with a Trace-positive Ultra result. A number of people in the communities with asymptomatic TB have been captured as MTB Trace by Xpert MTB/RIF Ultra and on repeat of the test it’s a negative result(1). TOP assay may confirm the status of this paucibacillary TB population. The significance of confirming paucibacillary TB with low assay signal will need to be evaluated clinically and

may assist by improving early detection in attaining the WHO TB control target of 2035 since there will be timely diagnosis and treatment to block transmission at the earliest stage.

1.3 General objective of the study

- To evaluate the diagnostic accuracy of TOP's 3-pon A diagnostic primer in the sputum of individuals with paucibacillary TB.

1.3.1 Research questions

What is the Diagnostic sensitivity of TOP assay (what proportion of Ultra positive samples with paucibacillary TB will be positive by TOP's 3-pon A diagnostic primer?

What is the diagnostic specificity of TOP Assay (what is the prevalence of TOP positive among the Ultra-negative samples)?

What is the diagnostic performance of TOP assay with low semi-quantitative Xpert Ultra results?

What proportion of Ultra Trace-positive samples remain positive when tested with TOP's 3-pon A diagnostic primer during the follow-up period?

1.3.2 Specific objectives of the study

1. To determine the diagnostic sensitivity of TOP assay (the proportion of Ultra positive samples with paucibacillary TB which are positive by TOP's 3-pon A diagnostic primer).

2. To determine the diagnostic specificity of TOP assay (the prevalence of TOP positive among Ultra-negative samples).

3. To determine the diagnostic performance of TOP assay with low semi-quantitative Xpert Ultra results.

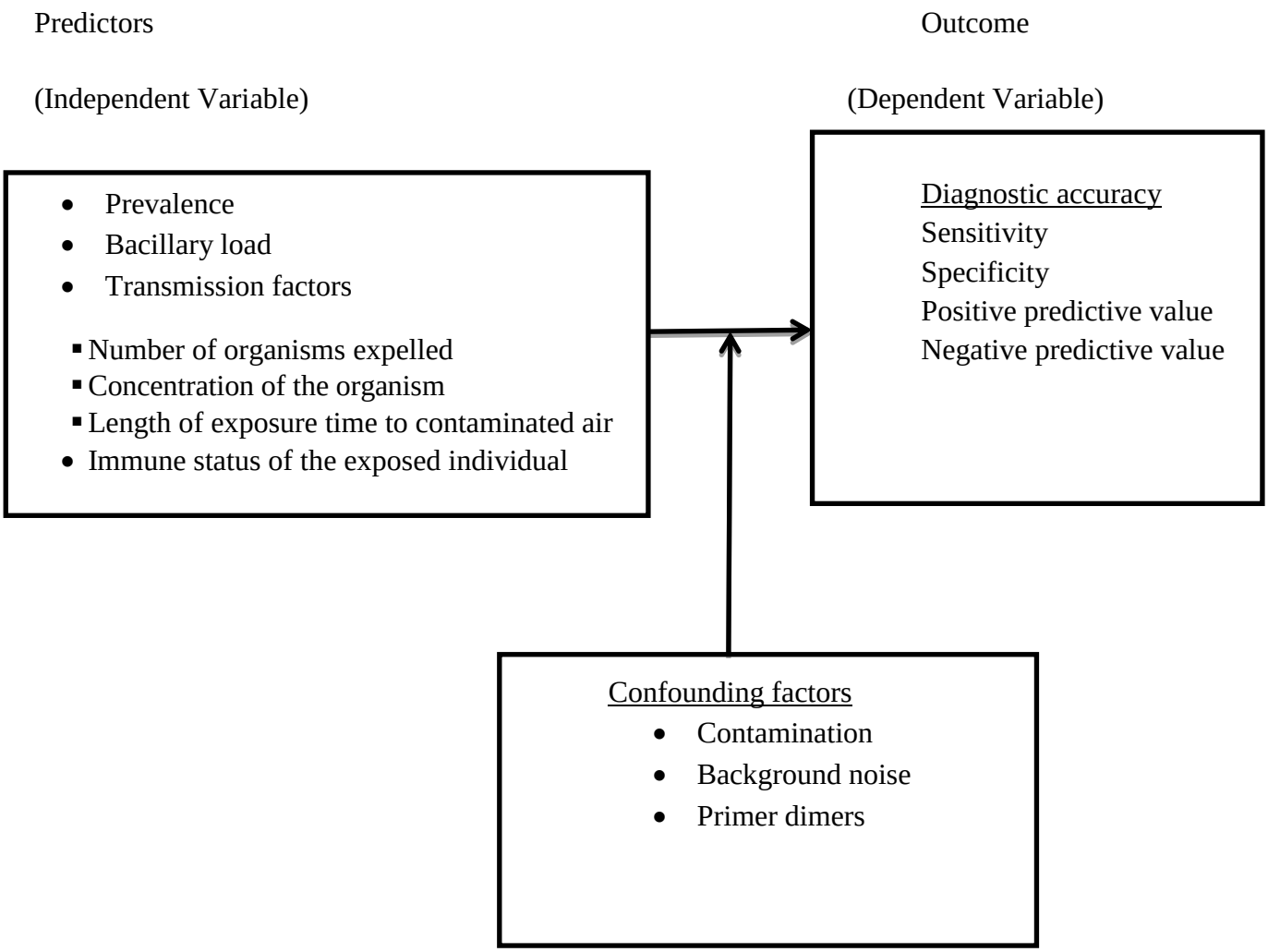
4. To determine the proportion of Ultra Trace-positive individuals who remain TOP positive on subsequent samples that are Ultra negative.

1.6 Justification and significance of the study

In Uganda, Xpert MTB/RIF Ultra has been used for patient care since it was authorised by WHO however it gives trace results which require further interpretation, people with Trace-positive results have been started on treatment yet some of them have turned to be false positive by culture which is the gold standard(21) and also negative on the subsequent sample collected, TOP assay has been developed with high sensitivity and highly specific to Mycobacteria in the MTB complex that enables detection of MTB at the lowest detection limit compared to Xpert MTB/RIF Ultra. In comparison with culture, TOP TB assay has sensitivity close to culture yet it is less prone to contamination and has a shorter Turnaround time hence cubing down on the transmission or progression to active TB. The use of TOP TB assay with higher sensitivity will explore diagnostic DNA sequences for early TB detection compared to Xpert MTB/RIF Ultra(13). The project aimed at studying people with low Xpert Ultra semi quantitative results on TOP assay to find out the proportion of people that remain positive with TOP TB assay overtime which will show evidence of MTB however the proportion of Xpert Ultra trace people who will be negative on TOP will be further evaluated based on the composite to assess whether they are false positive or not. The project will contribute to diagnostic development of the test which may offer true early TB diagnosis capturing TB at that non-transmission level. The initial test used colorimetric capture probe detection in a 96 well ELISA type plate and this study used gel electrophoresis for the detection of amplicons.

1.7 Conceptual Framework

Figure 1 summarizes an overview of the research concept



Key to the figure: MTB=Mycobacterium tuberculosis, TOP=Totally Optimized PCR

Figure 1: Conceptual framework

The outcome test results from the TOP assay are the dependent variables while the independent variables are the factors that affect the test performance for the participants with paucibacillary TB such as the number of organisms expelled, concentration of the organism, length of exposure time to contaminated air and immune status of the exposed individual, contamination, background noise and primer dimers are the cofounding factor which affect the outcome results.

CHAPTER TWO: LITERATURE REVIEW

2.1 Background- Current Burden

TB (Tuberculosis) is a communicable disease that is among the leading killers globally currently ranking above HIV/AIDS. It typically spreads through aerosols where its expelled into air thereafter infecting other healthy individuals bringing to about a quarter of the world's population infected by the bug(22).In 2020, the COVID-19 pandemic dislodged TB from the top infectious disease cause of mortality globally. Notably, global TB control efforts were not on track even before the advent of the COVID-19 pandemic. Many challenges remain to improve sub-optimal TB treatment and prevention services(23).TB cases are reported mostly in the regions of South-East Asia (44%), Africa (24%) and the Western Pacific (18%), with smaller percentages in the Eastern Mediterranean (8%)and Americas with Europe standing at (3%)(24). In Africa, current trend predictions were compared to the reductions needed to reach the WHO End TB targets and a few high burden countries are on track of achieving the 2020 milestones including Ugandan's neighbouring countries that is Tanzania and Kenya With the limited access to health care intervention available to cab down the high numbers of people living with Latent TB who are at risk of transgressing to active TB, the UN target of treating 40 million people with TB by 2022(25). However, this goal has been hampered by a combination of under reporting of detected cases and under diagnosis hence people with TB do not access health care or is not diagnosed when they do. WHO guidance issued in 2018 recommended TB preventive treatment for PLHIV, household contacts of bacteriologically confirmed pulmonary TB cases and clinical risk groups such as those receiving dialysis.

2.2 TB Pathogenesis

A detailed understanding of the crosstalk between human host and the pathogen *Mycobacterium tuberculosis* (Mtb) is vital. Principally, *Mycobacterium tuberculosis* enormous success is based on three capacities that is reprogramming of macrophages after primary infection/phagocytosis in order to prevent its own destruction, initiating the formation of well-organized granulomas, comprising different immune cells to create a confined environment for the host to pathogen standoff and the capability to shut down its own central metabolism, terminate replication and thereby transit into a stage of dormancy rendering itself extremely resistant to host defences and drug treatment(4). Primary infection can progress towards the active disease, be contained as latent infection (LTBI), and may also

be eradicated by the host's immune system. Less than 10% of infected individuals develop active TB during their lifetime. It is impossible to predict who can contain latent infection throughout lifetime and remain healthy, and who will develop active TB at some point. However, the risk of active disease is increased in immuno-compromising situations such as during anti-tumor necrosis factor (TNF) therapy of patients with chronic inflammatory diseases, by diabetes/obesity or by co-infection with human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) (26). Multiple factors are likely involved in defining overall risk of TB and the genetic make-up of both host and pathogen play a decisive role. Thus, biomarkers that would allow prognosis of TB reactivation in healthy individuals with LTBI would be of tremendous value(27).

2.3 Approach to diagnosis of TB

While advances in tuberculosis treatment continue to be made, patients remain undiagnosed and thus untreated. Advance in techniques for the diagnosis of tuberculosis are also being made in an attempt to address this problem(28, 29). Finding a microbiological diagnosis of tuberculosis especially in HIV patients is still challenging, despite recent developments in fast diagnostics. Current tuberculosis detection by laboratory testing may be less accurate and reliable in these patients due to sputum production issues and/or paucibacillary diseases.(30)Diagnosis of TB is based on a compatible TB history including history of smoking, diabetes contact with TB and positive TB tests. TB irrespective of body organ affected presents with constitutional symptoms of fevers, weight loss, and loss of appetite and night sweats. Other symptoms depend of site affected(29). For pulmonary TB (PTB), the most common and transmissible form of TB, presents with one or more of respiratory of symptoms of cough, chest pain, haemoptysis and dyspnea. Specific respiratory symptoms of more than two weeks are most suggestive of TB although among those who are immuno-suppressed, such as HIV patients, any of these symptoms for any duration are suggestive of TB(31). After the patient's history, has been taken and a physical exam has been performed, investigations are carried out depending on availability of the tests and the TB site. In pulmonary TB sputum specimens are examined using microscopy for the TB bacilli, MTB culture or using a new molecular test called Xpert MTB/RIF Ultra test(32)

2.4 Microscopy

TB diagnosis in sputum smear microscopy is performed by counting the number of bacilli present in approximately 100 fields. This counting can be done manually or automatically.

Two techniques are used for TB diagnostic with sputum smear microscopy that is Fluorescence microscopy and conventional microscopy(33). The greatest difficulty in diagnosis of TB and other mycobacterial infections by sputum microscopy is the test's lack of sensitivity and specificity however the sensitivity of the test has been improved considerably with improved techniques and standardisation of sputum preparation such as liquefaction with NALC and 2% sodium hydroxide and use of Auramine- rhodamine with the fluorochrome method instead of the classic acid-fast stains of ZN and Kinyoun, which use carbol-fuchsin(34). The sensitivity of Microscopy remains low (35% to45%) compared to MTB culture.

2.4.1 Conventional microscopy

It uses the carbol-fuchsinZiehl-Neelsen (ZN) or Kinyoun acid-fast stains, the primary diagnostic strategy for active tuberculosis (TB) worldwide that is recommended by the WHO(35). This method is constrained by its reliance on human skill and time-intensive nature. In developing countries, fewer skilled technicians, inadequate equipment and high caseloads compound the problem of numerous samples at limited microscopy facilities. Reading of three initial smears and viewing of 100 high-power fields per slide has always been recommended. This takes at least 15 min for experienced staff and the mental concentration and visual strain limits the volume of slides handled per day(36). Automated detection of acid-fast bacilli could hasten diagnosis, enhance quantitative and reduce on the turnaround timefor reporting of smear results in the health facility.

2.4.2 Fluorescence microscopy

It uses an acid-fast fluorochrome dye (e.g., Auramine 0 or Auramine- rhodamine). While the former uses an intense light source, such as a halogen or high-pressure mercury vapour lamp, the latter uses a conventional artificial light source(35). Fluorescence microscopy is on average 10% more sensitive than conventional microscopy however its main shortcoming are high costs of microscopy unit and its maintenance compared with the conventional microscopy unit. The handling and maintenance of fluorescence microscopy require advanced technical skills(33). In developed countries, fluorescence microscopy, rather than conventional microscopy, is the standard diagnostic method. Fluorescence microscopy is credited with increased sensitivity and lower work effort, but there is concern that specificity may be lower. Fluorescence microscopy is not often available in developing countries due to the high cost of the fluorescence microscope and, especially, that of its maintenance (37)

2.5 Xpert MTB/RIF Ultra assay

Xpert MTB/RIF Ultra is a rapid, highly sensitive nucleic acid amplification test (NAAT) with additional rifampicin susceptibility testing (15).It was developed to improve TB and Rifampin resistance detection which was accomplished by automating most of the steps required to process clinical samples. GeneXpert is a hemi nested PCR to amplify the rifampin resistance-determine region (RRDR) of the MTB rpoB gene. The specific molecular beacons are then used to detect both the presence of MTB and mutations responsible for approximately 95% of RIF-R(38). Despite its high sensitivity in tests of smear-positive sputum specimens, it's less sensitive when testing smear-negative sputum. The Xpert MTB/RIF assay was considered as a game changer due to its improved turnaround time since the results can be obtained within 2 hours, depending upon the timings of sample receiving and reporting of the result(39). The Xpert Ultra produces semi-quantitative readouts which include high, medium, low, very low and ''trace'' (40). The "trace" means that only the IS1081 and IS6110 targets were detected, but not the TB-specific regions in the rpoB gene however if a subsequent sample is tested from the sample patient, most of the times its negative which requires further interpretation. The sensitivity of Xpert MTB/RIF Ultra assay approaches the sensitivity of MTB culture.

2.6 MTB Culture

Cultivation of *M. tuberculosis* is the reference standard for TB diagnosis and treatment monitoring. Before culture, sputum samples must be decontaminated with reagents to reduce the growth of other bacteria and fungi that would otherwise outgrow the slowly growing *M. tuberculosis*. Decontamination with *N*-acetyl-L-cysteine combined with sodium hydroxide (NaOH), usually performed for about 15 minutes , is the most recommended method(41). However, previous clinical studies have shown that NALC-NaOH treatment reduces the viable *M. tuberculosis* count on solid culture and increases the time to positivity in liquid culture. The concentration of NaOH in order to eliminate all sputum contaminants is 2% which could result in a rate of negative *M. tuberculosis* culture results higher than that obtained with the standard concentration of 1%, confirming the detrimental effect of NaOH on *M. tuberculosis* viability(42). Phenotypes of *M. tuberculosis* which do not grow in routine culture media without the use of resuscitation-promoting factors (rpf's) are increasingly being recognized (9). These emerging reports provide important evidence that detection of all subpopulations of *M. tuberculosis* in patient specimens may not be achieved using culture techniques or NALC-NaOH decontamination-dependent tests.

2.7 Molecular bacterial Load Assay

The molecular bacterial load assay (MBLA) is a molecular test for the detection of viable *M. tuberculosis* bacilli. It is a reverse transcriptase quantitative PCR (RT-qPCR) that quantifies the *M. tuberculosis* load from patient sputum using the 16S rRNA gene as a reference gene. In contrast to culture, MBLA is rapid, sensitive, and specific and does not require an NALC-NaOH decontamination step(14). Unlike mRNA, which occurs in a low copy number and is exquisitely sensitive to degradation, the higher abundance and relative stability of rRNA make MBLA a more sensitive and robust test. MBLA was recently acknowledged to be a potential biomarker for TB treatment response monitoring, replacing culture and smear microscopy. Previous studies using non decontaminated sputa showed that MBLA has a higher sensitivity than culture (43).

2.8 Totally Optimized PCR (TOP) TB assay

TOP TB assay is a nucleic acid amplification test which utilises a combination of efficient sample processing novel gene target selection, modern primer design techniques, and an extended PCR for selective target isolation and amplification. Previous studies have shown that TOP assay has higher sensitivity than culture. The assay is highly specific for MTB and therefore is not affected by background genomic noise, which enables detection with heightened sensitivity. The assay targets a gene (ponA1) involved in the assembly of peptidoglycans in the MTB bacterial wall (18). The assay's diagnostic primer set 3-ponA-F/R targets sequences unique to all species in the MTB complex.

Culture has been used for long time as the gold standard for the diagnosis of TB however it has been problematic due to the long turnaround time (TAT), inherent degradation in the viability after decontamination of samples prior to culture and sensitivity of cultures is between 80-85% when compared to the TOP TB assay(17).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study design

This was a nested cross-sectional study in an 0n-going Trace Ultra Result iNsight in TB screening (TURN-T B) study, HDREC no. 901 evaluating and following a community-based cohort of people with Ultra Trace-positive sputum, in order to clarify their current burden and future risk of TB, while gaining insight into the disease dynamics that underlie their Ultra results. The study is structured as a baseline case-control study comparing Trace-positive individuals to positive and negative controls and as longitudinal cohort study with TB negative controls. The mother study follows up participants for 24 months, at 1 month, 3 months, 6 months, 12 months, and 24 months.

The current study evaluated the agreement between Totally Optimised PCR(TOP) assay and Xpert Ultra having low semi-quantitative results , and Xpert Ultra-negative samples obtained from participants enrolled in TURN-TB study.

3.2 Study site

Trace Ultra Result iNsight in TB screening (TURN-T B) study recruited participants from Kampala, Uganda and enrolment took place within Makindye division of Kampala where community wide TB screening within Kisugu, Wabigala and Bukasa (Namuwongo area) parishes was conducted. All the Ultra Trace-positive, Ultra-positive and Ultra-negative samples obtained from consented enrolled participants from Turn-TB study were stored in -80ºC freezer located in Mycobacteriology (BSL-3) Lab. The proposed study carried out TOP assay batch testing of samples from Genomics, Molecular and Immunology laboratories under Makerere University Biomedical Research Centre (MAK-BRC). The labs are located on third floor of pathology building at Makerere University College of Health Sciences, Mulago.

3.3 Study population

This study considered Xpert Ultra positive samples with low semi-quantitative results, and Xpert Ultra-negative samples (n=71) that were collected in TURN-TB study, the mother study consented participants ≥ 15years in Kampala, Uganda who participated in community-based TB testing using the Ultra assay on expectorated sputum. This study population included an Ultra positive population with MTB detected low, Very low and “Trace” and a negative control population. The mother TURN-TB study was involved in intensive

activities collecting sputum from adult residents in the study area through a combination of door-to-door screening, facility-based screening and contact investigation.

3.3.1 Inclusion criteria for the proposed study

This study used stored raw sputum samples collected from consented participants enrolled in the mother TURN-TB study of age ≥ 15 years who tested Xpert Ultra positive Low, Very, Trace ,and Ultra-negative with Gene Xpert Ultra assay, 2 positive controls (H37RV) and 2 negative controls (artificial sputum).

3.3.2 Exclusion criteria for the proposed study

This study excluded samples collected from active TB patients who were initiated on TB treatment before the community screening by the mother TURN-TB study and also the samples that had high TB bacilli load were excluded.

3.4 Sampling

3.4.1 Sample size Estimation

Out of 86 samples that were stored in the -80ºC freezer by the TURN-TB study, 71 samples were selected for inclusion in this study by convenience sampling purposively due to availability and the sample size was determined based on the finite population formula below (44)

Where, $$n = \frac{N}{\left[1 + N\left(e^2\right)\right]}$$

n=corrected sample size,

N=Population size

e=Margin of error, e=0.05 based on research condition

So, $$n = \frac{86}{1+86x\ (0.05)^2}$$ n = 71

3.5 Study Variables

3.5.1 Independent Variables

The independent variables were other factors that may have affected the TOP assay positivity which included bacillary load in the sample, transmission factors, number of organisms

expelled by the patient, concentration of the organism, and length of exposure time to contaminated air and immune status of the exposed individual.

3.5.2 Dependent Variables

Totally Optimised PCR (TOP) TB assay results which were either positive or negative and were used to calculate the positivity, sensitivity and specificity

3.6 Methodological details

Step by step procedure by which the study was executed

3.6.1 Summary flow chart

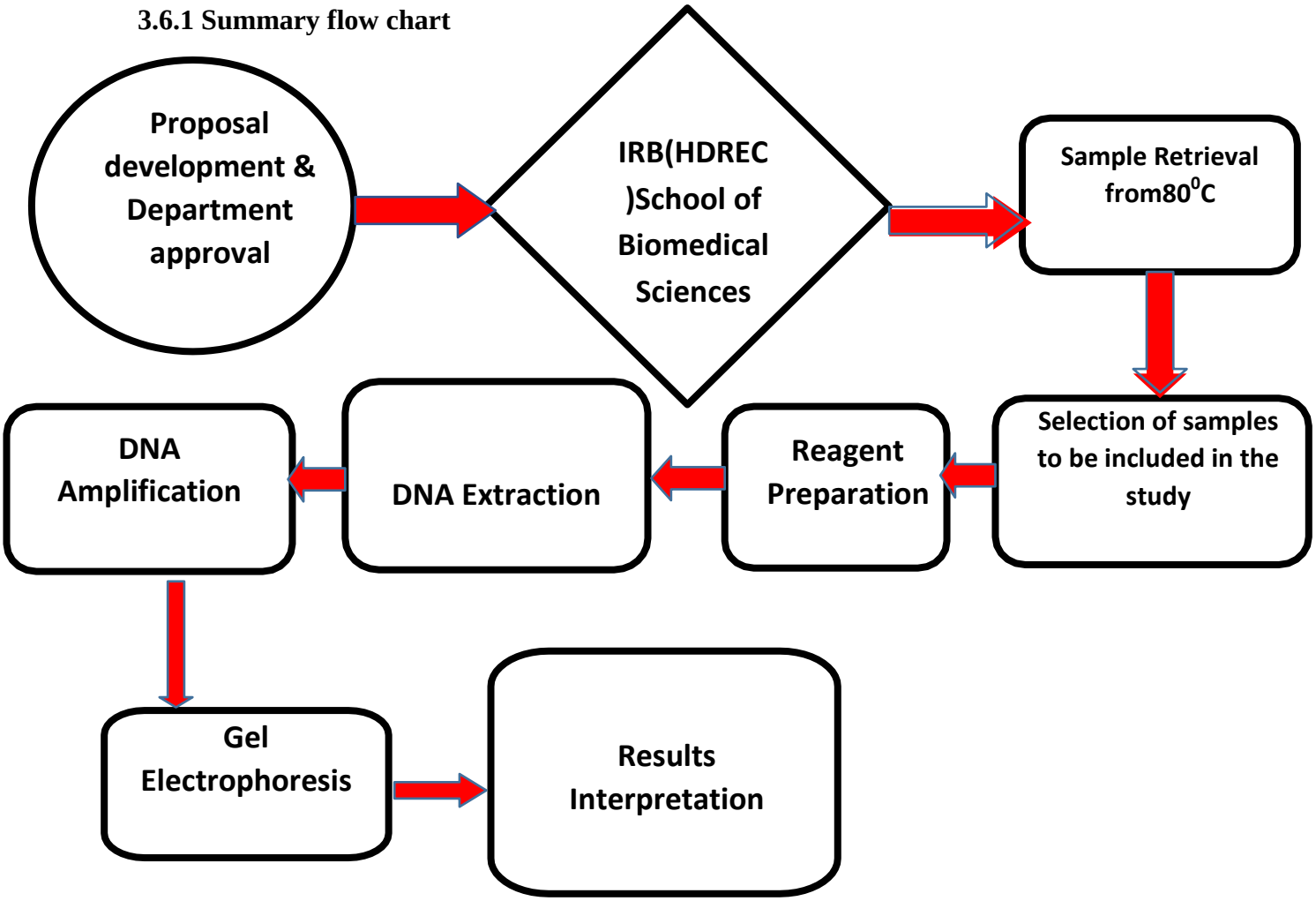


Fig 2: showing the summary flow chart of the study methodological details

3.6.1 Xpert MTB/RIF Ultra results

Samples that were collected from the study sites that is TB screening within Kisugu, Wabigala and Bukasa (Namuwongo area) were tested for Xpert MTB/RIF Ultra where all

MTB results were considered. All patients with Ultra Trace-positive results were requested to provide subsequent sputum samples after consenting for future specimen storage for testing on other more sensitive assays such as Totally Optimised PCR (TOP) TB assay. A portion of homogenised raw sputum sample was aliquoted in the cryovials and stored in -80 ºC freezer for TOP TB assay testing.

3.6.2 Totally Optimised PCR (TOP) assay

All stored raw sputum samples from patients with Xpert MTB/RIF Ultra Trace-positive, Ultra positive and Ultra negative results were retrieved from the freezer and thawed for TOP TB testing. The assay targets *Pon A1* gene involved in the assembly of peptidoglycans in the MTB bacterial wall. Two novel primer sets that target DNA sequences unique to *M. tuberculosis* in *ponA.* Primer set 3ponA-F/R amplifies only *M. tuberculosis*, and therefore avoids false-positive signals from other *Mycobacteria* and related species. Primer set 2ponA-F/Ra targets informative DNA sequences that distinguish M. *tuberculosis* from other closely related mycobacteria in the complex, and between strains of *M. tuberculosis*. Amplicons generated by 3-ponA were detected using gel electrophoresis.

3.6.3 Reagent preparation

3.6.3.1 Primer (Forward and Reverse) preparation

3 Pon A-F which contains 72.1nmoles was resuspended with 500µl of distilled water to make the stock solution, to avoid contamination; a working solution of 10 vials were aliquoted each containing 100µl (35µl amount of stock and 65µl of distilled water).

3 Pon A-R which contains 83.2nmoles was resuspended with 500µl of distilled water to make the stock solution, 10 vials were made each containing 100µl (30µl amount of stock primer solution and 70µl distilled water).

A 20µl PCR reaction was used which required 0.5µl of Forward and reverse primer, therefore a 100ul of working primer solution was enough to run 100/0.5 = 200 PCR reactions.

3.6.3.2 dNTPs preparation

Four dNTPs (dATP, dTTP,dCTP and dGTP) 100mM concentration each containing 250µl (25µmol) were mixed together to form 1ml containing 4x25=100µlmols. 9ml of distilled water was added to 1ml stock solution to form 10ml of the working solution, to avoid contamination, 20 vials were aliquoted each containing 0.5ml of working solution and stored in -80 freezer.

3.6.3.3 Preparation of Buffer 1 and Buffer 2

Buffer 1 contained 0.1M ascorbic acid and 30% hydrogen peroxide, 4.4075g of ascorbic acid was dissolved in distilled water, 33.3ml of hydrogen peroxide was added to the dissolved ascorbic acid and distilled water added up to 250ml to attain a pH of 4. The prepared bottle of buffer 1 was labeled with the date of preparation and the initial of the person preparing and stored at 4^{o}C.

Buffer 2 contained 1.0M NaOH and 0.01% Thymol blue, 10g of NaOH was dissolved in an ice bath, 25mg of Thymol blue was added and distilled water added up to 250ml at pH 10. The prepared buffer was labeled and store at room temperature.

3.6.4 TOP Assay sputum processing and DNA extraction

A three step color guided, sputum processing method was used that utilized the entire sample as follows;

i) *Homogenization:* 1ml of sputum sample was transferred to a 15ml tube, glass beads (0.1mm) were added to the sample, Buffer 1 (200µl <3 mL specimens and 400 µl for ≥3 mL) was added to the sample, this was to ensure efficient cell lysis during rapid vortexing, blood cell break down, chelation of iron, and inhibition of degrading enzymes; the vortexing was done until a foamy white homogeneous suspension was obtained.

ii) *Liquefaction:* Buffer 2 (200µl <3 mL specimens and 400µl for ≥3 mL) is a proprietary protein denaturation solution with NaOH that was used to liquefy the specimen for ease of pipetting and neutralization, addition of buffer B with heavy vortexing which gave a blue-green foamy solution, the sample was left to settle for 10mins until the sample separates into a foamy green upper part (supernatant) and a liquid blue part at the bottom which indicated successful digestion.

iii) *Single vial DNA purification and extraction:* 10µl of 2% Phenol RED was added to the stock 50ml of DNA extraction solution (Epicentre Biotechnologies, Madison, WI, USA), 50µl of the processed sample (liquid blue part) was placed into 200µl of DNA extraction solution and heated (<5 min) at 52^{o}C and then at 97^{o}C for degradation of proteins and other macro molecules. Extracted DNA was stored at -80°C until TOP PCR run to retain optimal DNA quality.

3.6.4 Quality Assurance and Quality Control during DNA extraction

Homogenization/ liquefaction was performed in the same container the specimen and the vial remains capped through the entire process and storage (minimizing risk of laboratory cross-

contamination). The use of low volume reagents (total volume 400-800µl per specimen) had the dual benefit of increasing sensitivity and, circumventing the need to concentrate the specimen by centrifugation, minimizing occupational risk.

3.6.5 PCR Amplification

PCR was performed using HotStarTaq DNA polymerase (Qiagen); 10x PCR buffer was used for the PCR reaction and a 20µl master mix was prepared. The denaturation was at 94ºC; the annealing temperature for 3-ponA PCR began at 61°C and ended at 59°C for 1 min, and elongation at 72°C for 45 sec. The PCR ran for 60 cycles.

Table 1: Thermo cycling for TOP Diagnostic primers (3-*pon*A-F and 3-*pon*A-R)

Step		Temperature (ºC)	Time	Cycles
Cycling 1	Denature	94	1min and 15s	1
Cycling2	Denature	94	45s	20
	Annealing	Gradient (-0.1 Co every cycle) 61 to 59	1min	
	Extension	72	45s	
Cycling 3	Denature	94	45s	40
	Annealing	59	1 min	
	Extension	72	45s	
	Final temperature	4	For ever	1

3.6.6 Gel electrophoresis

After amplification, the PCR products were visualized by electrophoresis (bio-vision UV electrophoresis machine) using 2% agarose gels stained with ethidium bromide. A 100 bp ladder was used and the gels ran at 120V and 300W for 30mins. PCR products were excised and purified (Quiagen gel purification) for storage and sent for sequencing at a later time.

3.6.7 Results interpretation

Samples were loaded from left to right across the top of the gel and were migrated vertically in lanes. Larger DNA molecules migrated slowly through the gel and were found at the top of the gel, while smaller DNA molecule migrated quickly through the gel and were found

towards the bottom. The bands contained stained DNA molecules embedded in gel and as the DNA molecules migrated through the gel, they were sorted according to their molecular weight, so that each band represented DNA of a specific molecular weight.

A 100bp ladder was included on either of the two sides which were used to determine the size of the each DNA molecule in the given sample.

Relative intensity of the bands was used to determine the relative abundance of the DNA molecules in a given sample because the amount of stain in a band is approximately proportional to the amount of DNA molecules in that band.

A dark or thick band indicated highly abundant DNA molecules in that sample and a faint or thin band indicated relatively small amount of DNA molecule in that sample.

3.7 Data Management and Analysis

This involved data collection, data cleaning, data analysis, data security and data dissemination.

3.7.1 Data collection

All laboratory data was collected in the laboratory book; this included the generated laboratory numbers, the different laboratory procedures and the results.

3.7.2 Data Cleaning and entry

All the laboratory data was proof read and reviewed with the supervisors before entry in the different software for accuracy. Data after cleaning was entered in Microsoft excel 2010; this data was again cleaned before it was transferred to statistical package for social scientist (SPSS) for analysis.

3.7.3 Data Analysis

Data was recorded in excel sheet, and then exported to SPSS and tables. A 2 by 2 table was used to calculate the diagnostic performance (sensitivity, specificity, positive and negative predictive values) of TOP assay against culture at 95% CI as indicated below.

Table 2: Diagnostic performance of TOP assay against Xpert Ultra and culture

		Culture	
		Positive	**Negative**
TOP Assay	**Positive**	a (No. of true positive)	b (No. of false positive)
	Negative	c (No. of false negative)	d (No. of true negative)

3.7.4 Data security

The data was stored in a password protected laptop and on addition; the data was kept with the supervisors to avoid loss of data in case of theft cases.

3.7.5 Data Dissemination plan

The study findings will be presented to the department of immunology and molecular biology at the school of biomedical sciences, college of health sciences of Makerere University. Also we shall share study findings with clinical staff of tuberculosis unit at Health centres where samples were collected. At least one copy of a book will be prepared for Albert cook and main library Makerere University. Also abstracts will be presented at both local and national conferences. Finally, a manuscript will be prepared and submitted to a peer review journal.

3.8 Study limitation

There was limited number of samples having Ultra trace results that could be included in the study.

3.9 Ethical considerations

The proposal was defended at Department of Immunology and Molecular Biology then at School of Biomedical Sciences Higher Degree Research Ethics Committee (SBSHDREC) for departmental and institutional review board approval respectively. Following the 124 convened meeting held on 15th December, 2022, the committee APPROVED the study

protocol and also granted approval of the research **SBS-2022-238**, which was valid for the period of 13/03/2023 to 13/03/2024. Kindly find attached approval letters in appendix section

CHAPTER FOUR: RESULTS

4.1 The Descriptive characteristics of participants

Table 3 describes characteristics of the participants. A total of 71 patient's samples were included in this study. The participants age range from 16 to 52 with an average age of 34 years and majority 53.52% (n=24) in the 16-30 age group. Most participants were males 52.11% (n=37). Table 4 describes the patients GeneXpert Ultra results and results obtained after testing with TOP by gender and the agreement between GeneXpert Ultra and TOP assay. For male participants, 61.11% (11 out of 18) tested positive with TOP assay, while 78.9% (15 out of 19) tested negative. Among females, 83.33% (15 out of 18) tested positive, and 81.25% (13 out of 16) tested negative. The agreement between the GeneXpert Ultra and TOP assay results was relatively high, indicating a reliable correlation between the two testing methods, regardless of gender.

Table 3 showing patients' socio-demographics

Characteristics	**Category**	**Frequency (n)**	**% percentage N=71**
Sex	Male	37	52.11
	Female	34	47.89
Age	Young adults (16-30)	38	53.52
	Middle age adults (31-45)	25	35.21
	Old age adults (>45)	8	11.27

Table 4 showing Assay characteristics by gender

Sex	Category	Xpert results	Xpert/TOP agreement	% Agreement
Male	Positive	18	11	61.11
	Negative	19	15	78.95
Female	Positive	18	15	83.33
	Negative	16	13	81.25

4.2 The diagnostic sensitivity of TOP assay (the proportion of Ultra positive samples with paucibacillary TB which are positive by TOP's 3-pon A diagnostic primer).

A total of 71 samples were included in this study, and prior testing with GeneXpert ultra had been performed on the same specimens, Of the 71 samples ,92.96% (66/71) samples yielded valid results when tested with the TOP assay, while 7.04%(5/71) produced indeterminate results. Among the 66 samples with valid TOP assay results, 45.46% (30/66) had undergone both sputum GeneXpert Ultra and culture testing. Comparing the TOP assay to sputum Xpert Ultra, the sensitivity of TOP assay was found to be 73.53% (n/N=25/34, 95% CI, 62.89-84.17), indicating that the TOP assay correctly identified 73.53% of the cases detected by sputum Xpert Ultra. Furthermore, the positive predictive value was determined to be 83.33%, 95% CI, 74.34-92.32) as shown in table 3 below. When compared to sputum culture, out of the seven (7) samples that tested positive using both GeneXpert Ultra and culture, the TOP assay successfully detected 71.43% (5/7) of them.

4.3 The diagnostic specificity of TOP assay (the prevalence of TOP positive among Ultra-negative samples).

A total of 32 Xpert Ultra-negative samples were analyzed. Among these samples, 27 were also negative on the TOP assay, while 5 out of 32 were detected positive by the TOP assay. The specificity of the TOP assay, when compared to Xpert Ultra, was determined to be 84.38% (27/32, 95% CI, 75.62-93.13) as shown in table 4, this indicates that the TOP assay correctly identified 84.38% of the cases that were negative according to Xpert Ultra. Among the patients who had negative results on both sputum Xpert Ultra and culture (a total of

12/30 patients), 66.67% (8/12) were also negative on the TOP assay, suggesting agreement between the TOP assay and the microbiological reference standard.

Table 4: Showing the diagnostic sensitivity and specificity of TOP assay

		Xpert		**Total**
		Positive	**Negative**	
TOP Assay	**Positive**	25 (No. of true positive)	5 (No. of false positive)	30
	Negative	9 (No. of false negative)	27 (No. of true negative)	36
	Total	34	32	66

True D defined as Xpert U_TRA =0			**[95% Conf. Inter.]**	
Sensitivity	Pr(+/D)	73.53%	62.89%	84.17%
Specificity	Pr(-/˜D)	84.38%	75.62%	93.13%
Positive predictive value	Pr(D/+)	83.33%	74.34%	92.32%
Negative predictive value	Pr(˜D/-)	75.00%	64.55%	85.45%
Prevalence	Pr(D)	51,52%	39.46%	63.57%

4.4 The diagnostic performance of TOP assay with low semi-quantitative Xpert Ultra results.

The diagnostic sensitivity of TOP assay consistently increased in patients with higher sputum mycobacterial loads, as indicated by the semi-quantitative results of sputum Xpert ultra. Specifically, among the 22 cases with Xpert Ultra Low results, 90.91% (20/22) were detected

by TOP, for the 11 cases with Very Low results, 45.45% (5/11) were detected by the TOP assay,. Finally, one case with a Trace-positive result was not detected by TOP as shown in figure 3 below.

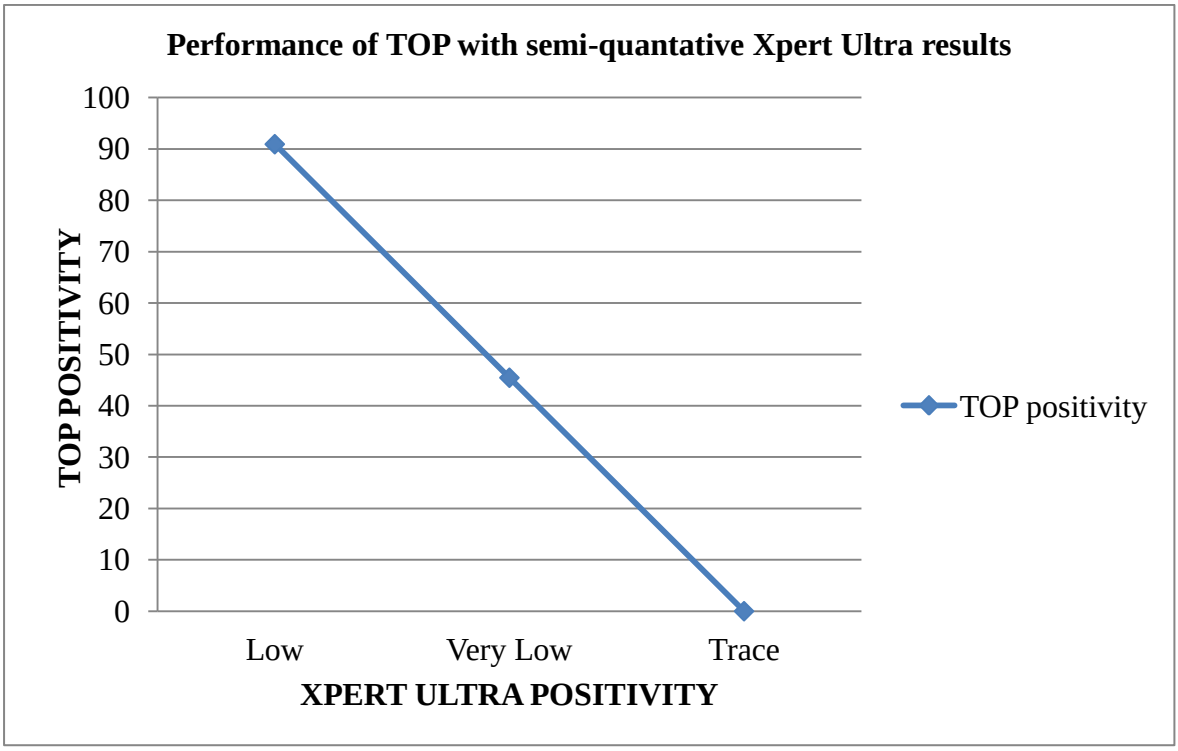


Fig. 3 illustrates the diagnostic performance of the TOP assay in relation to the grading of semi-quantitative Xpert Ultra results. This visual representation allows for a comprehensive comparison between the TOP assay and the different levels of Xpert Ultra results grading.

CHAPTER FIVE: DISCUSSION

Our study included a total of 71 samples, with 92.96% (66/71) yielding valid results when tested with the TOP assay. This high proportion of valid results suggests the robustness and reliability of the TOP assay in processing the samples. Furthermore, when compared to sputum Xpert Ultra, the TOP assay demonstrated a sensitivity of 73.53%, indicating that it correctly identified 73.53% of the cases detected by Xpert Ultra, however this sensitivity was lower than that demonstrated in a study carried out in Boston to evaluate further evidence of Mycobacterium tuberculosis in the sputum of culture negative pulmonary tuberculosis suspects using an ultrasensitive molecular assay (45). The pilot demonstrated feasibility and 100% (95% CI 85–100) sensitivity in predominantly smear-positive specimens; TOP's specificity against solid media culture was low (58%, 37–77) but improved against a CRS (93%, 68–100). And, when using the CRS, TOP (88%, 1–99) had greater sensitivity than solid or liquid media culture (25%, 3–65) and similar specificity (both 100%, 93–100).

It is important to note that in our study, the positive predictive value of the TOP assay was determined to be 83.33%, indicating the proportion of the positive TOP assay results that were true positives. This indicates the overall accuracy of the TOP assay in identifying true TB cases. These results are valuable in supporting the use of the TOP assay as a reliable diagnostic tool for paucibacillary TB cases. Our findings demonstrate that the TOP assay effectively addresses well-known challenges associated with obtaining sputum samples, such as inadequate volumes. Remarkably, the TOP assay can utilize as little as 0.5 or 1 ml of the sample, significantly improving diagnostic sensitivity. The TOP assay presents promising solutions by enabling the use of sputum for diagnosing paucibacillary TB. This break through eliminates the previous reliance on non-pulmonary clinical samples like blood, gastric aspirates, urine, and stool, which were previously deemed necessary for diagnosis.

Additionally, when comparing the TOP assay to sputum culture, our study found that the TOP assay successfully detected 71.43% of the samples that tested positive using both Xpert Ultra and culture. This finding suggests that the TOP assay has the potential to serve as a rapid alternative to culture testing in detecting TB cases, particularly in resource-limited settings where culture methods may not be readily available.

The specificity of the TOP assay, when compared to Xpert Ultra, was determined to be 84.38%, indicating that it correctly identified 84.38% of the cases that were negative according to Xpert Ultra; these results suggest that the TOP assay has a high level of

agreement with Xpert Ultra in indentifying true negative cases. Furthermore, among the patients who had negative results on both Xpert Ultra and culture, 66.67% were also negative on the TOP assay, indicating concordance between the TOP assay and the microbiological reference standard. However this finding showed a higher specificity compared to a proof of concept study done in 2016 in Mbarara, Uganda to detect and quantify *Mycobacterium tuberculosis* in the sputum of culture negative HIV infected pulmonary Tuberculosis suspects using TOP assay (17), they found out that using culture as reference, TOP had 100% sensitivity but 35% specificity**,** these results reinforce the use of the TOP assay as a reliable tool for ruling out TB in Xpert Ultra-negative cases, minimizing the risk of false-positive results and unnecessary treatments.

Our study demonstrated that the diagnostic sensitivity of the TOP assay consistently increased in patients with higher sputum mycobacterial loads, as indicated by the semi-quantitative results of Xpert Ultra. Specifically, among the cases with Xpert Ultra Low results, the TOP assay detected 90.91% of the cases, indicating its effectiveness in identifying TB cases even in situations with lower mycobacterial loads.. However, in cases with Very Low results, the sensitivity of the TOP assay decreased to 45.45%, suggesting its limitations in detecting TB in cases with very low mycobacterial burden. Furthermore, the TOP assay failed to detect one case with a Trace-positive result, indicating challenges in identifying TB in cases with minimal mycobacterial presence. These findings highlight the influence of mycobacterial load on the performance of molecular assays. They emphasize the need for cautious interpretation of results, especially in cases with lower Xpert Ultra grading. Integrating additional diagnostic approaches or confirmatory tests may be necessary to ensure accurate TB diagnosis in such cases.

CHAPTER SIX: CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Our research study provides valuable insights into the diagnostic performance of the TOP assay in detecting paucibacillary TB cases. The TOP assay demonstrated moderate sensitivity and specificity, particularly in cases with higher mycobacterial loads as indicated by Xpert Ultra grading. However, limitations are observed in cases with lower mycobacterial burden. Emphasizing the need for complementary diagnostic methods or confirmatory tests in such scenarios. By understanding the strengths and limitation of the TOP assay, health care providers can make informed clinical decisions and enhance TB control efforts.

Further research is warranted to explore ways to improve the sensitivity of the TOP assay in detecting TB cases with lower mycobacterial loads, Amplicons generated by 3-ponA to be detected using a capture-probe colorimetric assay as opposed to the gel electrophoresis used in this study to increase the sensitivity of the TOP. Additionally, the cost-effectiveness and feasibility of implementing the TOP assay in different health care settings should be investigated to assess its potential as a routine diagnostic tool. Overall, our study contributes to the existing literature and paves the way for future advancements in TB diagnostics.

6.2 Recommendations

- It is important to acknowledge the limitations of our study. The sample size was relatively small, and further research with larger sample sizes especially with Trace-positive Xpert results is warranted to validate our findings.
- The influences of various factors, such as age, co morbidities, and HIV status, on the assay's performance should be investigated.
- Consideration should be given to combining the TOP assay with other diagnostic modalities, such as culture-based methods or alternative molecular assays, to enhance overall diagnostic accuracy, especially in challenging cases with low mycobacterial loads.
- Conduct cost-effectiveness analyses to evaluate the feasibility and economic impact of implementing the TOP assay as a routine diagnostic tool in health care settings.

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APPENDICES

Appendix 1: IRB Approval

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COLLEGE OF HEALTH SCIENCES
SCHOOL OF BIOMEDICAL SCIENCES
RESEARCH ETHICS COMMITTEE

13/03/2023

To: Kenneth Mubiru

Makerere University
0701324215

Type: Initial Review

Re: SBS-2022-238: DIAGNOSTIC ACCURACY OF Mycobacterium.tuberculosis DNA AS DETECTED BY THE TOP ASSAY AMONG INDIVIDUALS WITH PAUCIBILLARY TB, 2.0, 2023-02-10

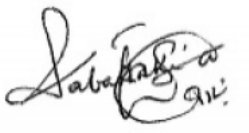
I am pleased to inform you that at the **124** convened meeting on **15/12/2022**, the MAK School of Biomedical Sciences REC (SBSREC), committee meeting, etc voted to approve the above referenced application. Approval of the research is for the period of **13/03/2023** to **13/03/2024.**

As Principal Investigator of the research, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the protocol or the consent form must be submitted to the REC for re-review and approval **prior** to the activation of the changes.
3. Reports of unanticipated problems involving risks to participants or any new information which could change the risk benefit: ratio must be submitted to the REC.
4. Only approved consent forms are to be used in the enrollment of participants. All consent forms signed by participants and/or witnesses should be retained on file. The REC may conduct audits of all study records, and consent documentation may be part of such audits.
5. Continuing review application must be submitted to the REC **eight weeks** prior to the expiration date of **13/03/2024** in order to continue the study beyond the approved period. Failure to submit a continuing review application in a timely fashion may result in suspension or termination of the study.
6. The REC application number assigned to the research should be cited in any correspondence with the REC of record.
7. You are required to register the research protocol with the Uganda National Council for Science and Technology (UNCST) for final clearance to undertake the study in Uganda.

No.	Document Title	Language	Version Number	Version Date
1	Protocol	English	2.0	2023-02-10

Yours Sincerely



Assoc Prof. Erisa Mwaka
For: MAK School of Biomedical Sciences REC (SBSREC)

Appendix 2: Administrative approval to use TURN-TB stored samples

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COLLEGE OF HEALTH SCIENCES
School of Biomedical Sciences
Department of Medical Microbiology

TURN-TB STUDY

3 November 2022

To: **Mr. MUBIRU KENNETH**
Makerere University
P.O. Box 7072, Kampala, Uganda.
Date: 31/OCTOBER/ 2022,

RE: Permission to use the TURN-TB raw sputum samples for Clinical Research.

Protocol title: **"DIAGNOSTIC ACCURACY OF *Mycobacterium tuberculosis* DNA AS DETECTED BY THE TOP ASSAY AMONG INDIVIDUALS WITH PAUCIBILLARY TB"**

You are permitted to use sputum specimens banked from the TURN-TB study by the Mycobacteriology laboratory, Makerere University, along with accompanying clinical and laboratory data, for **Clinical Research** (clinical and laboratory data) for the above mentioned masters thesis project. Permission is granted for a period of 1-year from the date of this letter. The samples to be accessed should not exceed 250 samples.

The above permission is granted to you on the following conditions: -

1) You should obtain final approval of the protocol and all accompanying documents from the from Research Ethics Committee (REC) and the Uganda National Council for Science and Technology before starting the study.

2) Your human subjects research training should be completed and up to date when working with these specimens and accompanying clinical data.

3) The TURN-TB study team should be kept informed of your progress on this protocol.

4) Any publications resulting from this work should be reviewed and approved by the TURN-TB PI.

5) You will adhere to the approved Risk Management Plan during the implementation of the study.

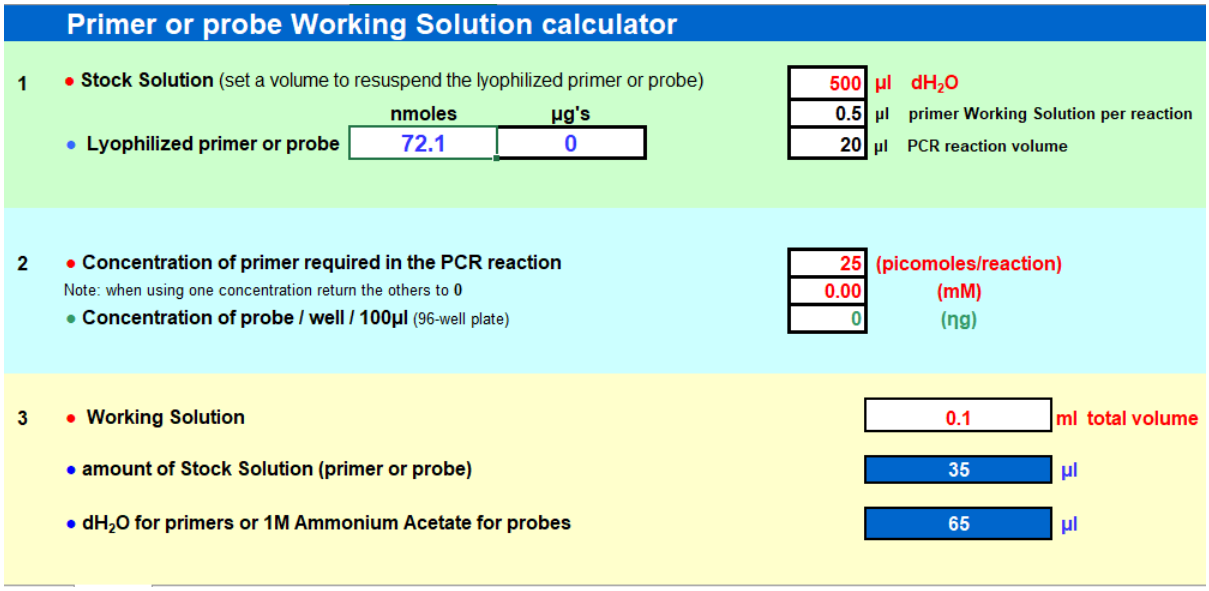
For any further enquiry or questions, the REC may contact me via email at ekendall@jhmi.edu.

Sincerely,

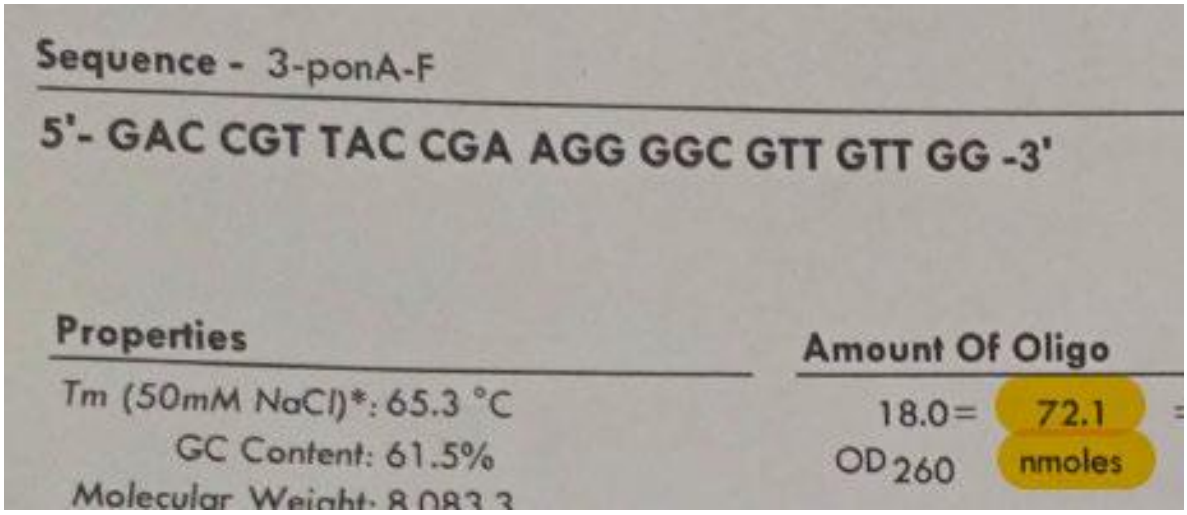
Dr. Emily Kendall

Appendix 3: Primer working solution preparation

Primer preparation. Primer name	nmoles synthetized	Resuspend lyophilized primer dH2O (µl)	Primer working solution per reaction (µl)	PCR reaction volume (µl)	picomoles of primer per reaction	volume of primer working solution (ml)*	Amount of stock primer solution (µl)	dH2O (µl)
3-ponA-F	72.1	500	0.5	20	25	100 ul (0.1)	35	65
3-ponA-R	83.2	500	0.5	20	25	100 ul (0.1)	30	70



Primer working solution calculator for 3-pon A-f



Primer or probe Working Solution calculator

1 • **Stock Solution** (set a volume to resuspend the lyophilized primer or probe)

	nmoles	µg's
• Lyophilized primer or probe	83.2	0

500	µl	dH_2O
0.5	µl	primer Working Solution per reaction
20	µl	PCR reaction volume

2 • **Concentration of primer required in the PCR reaction**

Note: when using one concentration return the others to **0**

• **Concentration of probe / well / 100µl** (96-well plate)

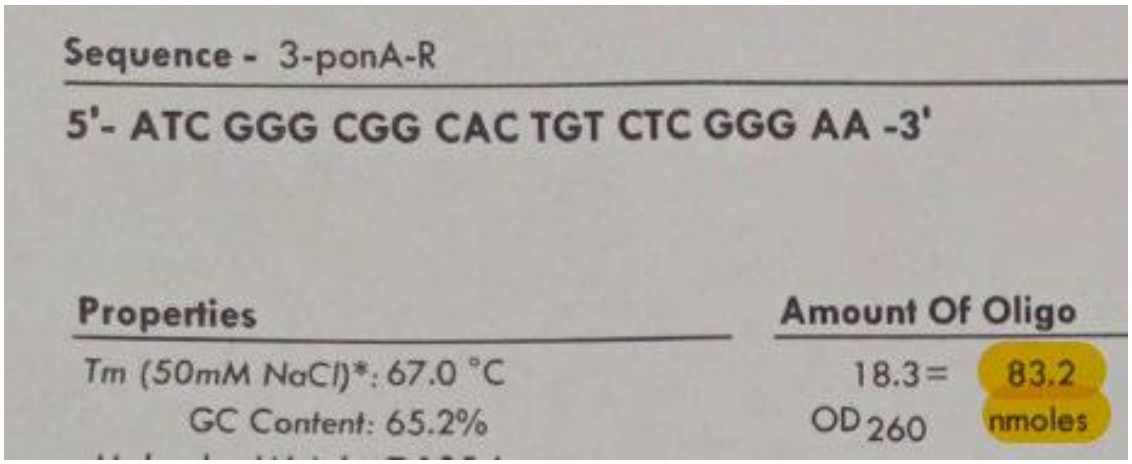
25	(picomoles/reaction)
0.00	(mM)
0	(ng)

3 • **Working Solution** 0.1 ml total volume

• **amount of Stock Solution (primer or probe)** 30 µl

• **dH_2O for primers or 1M Ammonium Acetate for probes** 70 µl

Primer working solution calculator for 3-pon A-R



Appendix 4: Making master mix PCR (20µl) reaction TOP diagnostic primers (3-ponA-**F and 3- ponA-R)**

Reagent	**Stock concentration**	**Volume in µl**
PCR buffer	10X	1.8
dNTP	100Mm	2
$MgCl_2$	25Mm	0.2
Primer- F	50nmole	0.5
Primer-R	50nmole	0.5
DNA polymerase	1000U	0.5
RNA free water	500ml	11
DNA sample		4
Total		20