

**IMPROVING POINT-OF-CARE DIAGNOSIS OF
TUBERCULOSIS: DEVELOPMENT AND EVALUATION
OF NOVEL TECHNOLOGIES**

by

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SUBMITTED TO THE UNIVERSITY OF CAPE TOWN

In fulfilment of the requirements for the degree

DOCTOR OF PHILOSOPHY

in the department of

PATHOLOGY

(MEDICAL MICROBIOLOGY)

FACULTY OF HEALTH SCIENCES

Submitted December 2016

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Dedicated to all the patients who have succumbed to tuberculosis.

“Yet the captain of all these men of death that came against him to take him away was the consumption, for it was that that brought him down to the grave.”

John Bunyon, *The Life and Death of Mr. Badman*, 1680

DECLARATION

I, Vineshree Mischka Moodley, hereby declare that the work on which this dissertation/thesis is based is my original work (except, where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ABSTRACT

With an estimated third of all tuberculosis (TB) cases being missed, the need to develop rapid, simple and accurate diagnostic tests is critical. The last five years has seen an unprecedented activity in the development of a range of new tests. However, a major concern is that not all marketed TB tests have been assessed rigorously, particularly in terms of diagnostic accuracy, robustness under operational conditions in the field, and practical usefulness. This dissertation comprises a compilation of diagnostic clinical studies of novel point-of-care tests, namely a chemiresistive “TB breath-analyser”; a lipoarabinomannan (LAM) urine dipstick, and an adaptation of the Xpert®MTB/RIF assay for use on blood. Lastly, there is a modification of the sputum collection device (SCD) to enable specimen processing without the requirement of a biosafety cabinet. The chemiresistive sensor, which detects volatile organic compounds released by *Mycobacterium tuberculosis* in a patient’s breath, demonstrated a high sensitivity (100%) and specificity (92%) for distinguishing patients with active TB from healthy controls. However, sensitivity (74%) and specificity (63%) were lower when the culture-negative participant group was compared to the culture-positive participants. The test shows potential as a useful screening test for TB with further refinement of the sensor technology. The LAM dipstick was shown to be useful in hospitalised HIV-infected patients with CD4 T-cell counts <200 cells/ μ L reinforcing the data from other studies. Although the blood Xpert®MTB/RIF assay showed some utility in diagnosis of TB in hospitalised patients with very advanced HIV, given the poor sensitivity and specificity, and the requirement for specialised equipment as well as a large volume of blood for testing, it is unlikely that Xpert®MTB/RIF testing on blood will contribute much over other existing diagnostics in resource-

limited settings. Finally, the redesigned SCD offers a solution to biosafety concerns with minimal impact on patient acceptability and clinical care.

ACKNOWLEDGEMENTS

I wish to acknowledge the following individuals:

- my supervisors, Prof. Mark Nicol and Dr Susan Dorman, for allowing me to participate in their research projects as well as for their support and guidance;
- my collaborators, which includes Dr Will Royea and Vince Martin of NextDimension Technologies; Prof. David Kelso from Northwestern University; the TBCDRC team including Rachel Kubiak and Aletta Bareng Nonyane; Dr Padmapriya Banada and Dr David Alland;
- the study support staff of the various projects based at the University of Cape Town, including Widaad Zemanay, Slindile Mbhele, Gaironesa Omar, Khunji Khume, as well as the microbiology staff at the National Health Laboratory Services C18 TB laboratory at Groote Schuur hospital, Cape Town;
- the Discovery Foundation for their award of a Discovery Academic Fellowship;
- the funders of the different projects: Bill and Melinda Gates Foundation, PATH, and the TB Clinical Diagnostics Research Consortium (TBCDRC);
- Dr Yoshan Moodley for assistance with some of the statistical aspects of the dissertation and proof-reading of chapter three;
- the participants of the various studies;
- and finally, my family and friends, in particular Graham, Skyler and my parents, for their love and support.

PREFACE

The following constitutes the PhD candidate's contributions to the various studies.

Chapter two: A field evaluation of a chemiresistive sensor array for the rapid diagnosis of pulmonary tuberculosis

- One of the principal investigators of the study.
- Contributed to protocol development and literature search.
- Obtained ethical approval as well as approval from the Department of Health (DoH)/City of Cape Town.
- Involved in compiling study budget.
- Devised consent forms.
- Devised clinical research forms (CRFs).
- Devised standard operating procedures.
- Implementation of study.
- Performed clinical testing of device.
- Training of additional staff.
- Troubleshooting problems with software as well as instrument and sensor.
- Performed data entry and collated all data (patient data, sensor readings, microbiology results).
- Quality control of CRFs as well as sensor readings obtained (from clinical research workers).
- Data synthesis and data analysis.
- Oral presentation of data at ECCMID; 2012, London and FIDSSA; 2011, Durban. Poster presentation at UCT Health Sciences Faculty Research Day, 2011.

Chapter three: Feasibility of using the Determine™ lateral flow lipoarabinomannan test for the diagnosis of tuberculosis (TB) in HIV/TB co-infected participants in Cape Town, South Africa

- One of the principal investigators of the study.

- Contributed to local site protocol development.
- Devised study budget.
- Obtained local ethical approval as well as approval from the DoH/City of Cape Town.
- Contributed to manual of procedures.
- Advised on development of CRFs for local study site.
- Backup reader of urine LAM dipsticks.
- Implementation of the study.
- Day-to-day management of clinical trial.
- Training of staff members.
- Contributed to quality assurance procedures.
- Data collation of local site.
- Data synthesis and data analysis.
- Contributed to write-up of two published manuscripts as well as poster presented at 19th Conference on Retroviruses and Opportunistic Infections; 2012, Washington.

Chapter four: diagnostic performance of the Xpert®MTB/RIF assay on blood specimens from HIV-infected participants with suspected tuberculosis

- One of the principal investigators of the study.
- Contributed to local site protocol development.
- Devised study budget.
- Obtained local ethical approval as well as approval from DoH/City of Cape Town.
- Advised on development of CRFs for local study site.
- Contributed to manual of procedures.
- Contributed to troubleshooting problems with test assay.
- Implementation of the study.
- Day-to-day management of clinical trial.
- Training of staff members.
- Contributed to quality assurance procedures.
- Data collation of local site.
- Data synthesis and data analysis.

Chapter five: Development of an integrated sputum collecting/processing device

- One of the principal investigators of the study.

- Developed protocol.
- Obtained ethical approval as well as approval from DoH/City of Cape Town. Provided feedback to DoH at closure of study.
- Devised consent forms.
- Devised CRFs.
- Implementation of the study.
- Performed clinical testing of device.
- Trained additional staff in performing testing.
- Performed data entry as well as data collation.
- Involved in quality control of CRFs.
- Data synthesis and data analysis.
- Contributed to design of new prototypes and the ultimate prototype that was selected for further development.
- Formulated formal feedback report to funder.

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LIST OF ABBREVIATIONS

ADA adenosine deaminase

AFB acid-fast bacilli

AIDS Acquired Immunodeficiency Syndrome

ART antiretroviral therapy

BCG Bacille Calmette-Guerin

cAMP cyclic adenosine monophosphate

CI confidence interval

CT computed tomography

CFU colony forming units

cm centimetre

CPA cross-priming amplification

CrI credible interval

CRW clinical research worker

CXR chest radiography

DNA deoxyribonucleic acid

DST drug susceptibility testing

EPTB extrapulmonary tuberculosis

ELISA enzyme-linked immunosorbent assay

FDG fluorodeoxyglucose

¹⁸F-FDG PET/CT ¹⁸F-fluorodeoxyglucose positron emission tomography/
computerised tomography

FM fluorescent microscopy

GC/MS gas chromatography/mass spectrometry

HIV human immunodeficiency virus

HPF high-power field

IgA immunoglobulin A

IgG immunoglobulin G

IgM immunoglobulin M

IGRA interferon gamma release assay

INH isoniazid

IPT isoniazid preventative therapy

IS insertion sequence

LAM lipoarabinomannan

LAMP loop-mediated isothermal amplification

LDA linear discriminant analysis

LED light emitting diodes

LFA lateral flow assay

LJ Lowenstein-Jensen

LPA s line probe assays

ManLAM mannose-capped lipoarabinomannan

MDR multidrug-resistant

MDR-TB multidrug-resistant tuberculosis

min minute

MGIT mycobacterial growth indicator tube

μL microlitre

mL millilitre/s

mm³ cubic millimetre

MODS microscopic observation drug susceptibility

MRI magnetic resonance imaging

MUT mutation

NAATs nucleic acid amplification tests

NPV negative predictive value

NTM nontuberculous mycobacteria

OD optical density

PAMP pathogen-associated molecular pattern

PCR polymerase chain reaction

PI phosphatidyl-inositol

PLWH people living with HIV

POC point-of-care

ppb parts per billion

ppt parts per trillion

PPV positive predictive value

PTB pulmonary tuberculosis

R resistance

RIF rifampicin

RNA ribonucleic acid

ROC receiver-operating characteristic

RRDR rifampicin resistance determining region

SESI-MS secondary electronspray ionisation-mass spectrometry

spp. species

SR sample treatment reagent

TAT turnaround time

TB tuberculosis

TST tuberculin skin test

UR uncertainty range

VOC volatile organic compound

WHO World Health Organisation

WHO-TB World Health Organization TB symptom screening algorithm

WT wild-type

XDR extensively drug-resistant

ZN Ziehl-Neelsen stain

LIST OF SYMBOLS

% per cent

\$ dollar (US)

> greater than

< less than

$\geq$ greater than or equal to

$\sim$ approximation

® registered trademark

™ unregistered trademark

°C degrees Celsius

β beta

Δ change

CHAPTER ONE: LITERATURE REVIEW

'Diagnosis is not the end, but the beginning of practice.'

Martin H. Fischer

1. INTRODUCTION

Tuberculosis (TB) has plagued humankind throughout much of known history.⁽¹⁻³⁾ It is postulated that the genus as represented by *Mycobacterium ulcerans* may have originated more than 150 million years ago.⁽⁴⁾ Modern techniques using genome sequencing have concluded that the modern progenitor was present at least three million years ago in East Africa and that our early hominid ancestor may already have been suffering from TB.⁽⁵⁾ The traditional belief was that *Mycobacterium tuberculosis* originated in animals and was transferred to humans during the domestication process in the so-called "fertile crescent".⁽⁶⁾ Comparative genomic and molecular marker analyses have since revealed that the *M. tuberculosis* complex has accumulated deletions over time (which can be used to distinguish individual species and lineages), and these analyses suggest a very different scenario, making the human strain, *M. tuberculosis*, the most ancient strain.⁽⁶⁻⁸⁾

Modern strains of *M. tuberculosis* are estimated to have emerged from a common ancestor approximately 20,400-15,300 years ago.⁽⁹⁾ The earliest known human TB was detected and characterised in samples from the

submerged Neolithic site of Atlit Yam, a 9,000-year-old settlement submerged in the sea off the coast of Haifa in Israel.⁽¹⁾ Archaeological evidence of TB infections dating back more than 5,000 years has also been found in Egypt.^(2, 10-12) Paleopathological changes (collapse of the vertebrae, periosteal reactive lesions and osteomyelitis) have been documented in Neolithic Sweden⁽¹³⁾, Neolithic Italy^(14, 15), the eastern Mediterranean⁽¹⁾, Iron Age South-East Asia⁽¹⁶⁾ and China.⁽¹⁷⁾ These data suggest the first epidemiological transition (Neolithic agriculture and permanent settlements) led to an increase in TB; with later increases in urban environments of the late medieval period.⁽¹⁸⁾

M. tuberculosis experienced an evolutionary bottleneck when it became an obligate human pathogen and developed a clonal relationship with different human lineages.^(19, 20) Subsequent co-evolution has resulted in the majority of TB infections being latent.⁽²⁰⁾ In the past eras of low human population density, *M. tuberculosis* adapted over time in response to host-adaptive changes and vice versa.⁽²⁰⁾ More virulent *M. tuberculosis* strains will attack their human host, killing the most susceptible and leaving the more resistant as survivors.⁽²⁰⁾ The development of antibiotics has shortened the mutualistic cycle significantly, but the combination of human immunodeficiency virus (HIV) co-infection, antimicrobial therapy and increased global human population density is altering this balance leading to the emergence of some *M. tuberculosis* strains that are not only more transmissible, but also more virulent.^(20, 21)

TB has surged in great epidemics and then receded. In Europe and North America, a decline in TB infections has continued to the present time, probably largely unaffected by public health programmes or effective chemotherapy.⁽²²⁾ The incidence of TB appears to have peaked around the middle of the 1700's in Great Britain where a temporal association has been

suggested between increased TB incidence and rapid industrialisation and urbanisation.^(23, 24) This likely resulted in increased transmission due to increased population density and crowded living conditions whilst poor nutritional status and other risk factors increased progression to active disease.⁽²⁴⁾ Throughout the 20th century, even before the introduction of anti-tuberculosis drugs, TB incidence decreased steadily due to the improvements in living conditions, economic growth, and social reform.^(23, 24) In other parts of the world, especially in sub-Saharan Africa and South-East Asia (Figure 1), where TB is fuelled by acquired immunodeficiency syndrome (AIDS)^(22, 25-27) and other factors such as poverty^(26, 27), crowded living conditions^(26, 27) and lack of access to effective, affordable health care services⁽²⁶⁻²⁸⁾; the TB epidemic continues unabated.⁽²⁶⁾

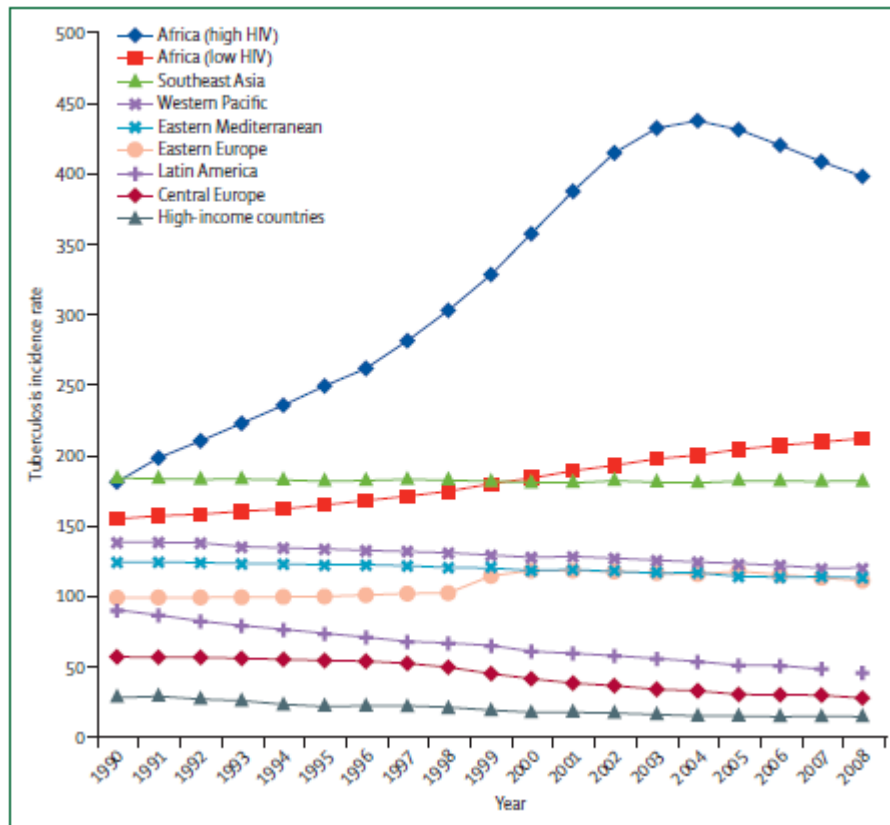


Figure 1. Trends in estimated TB incidence rates in nine subregions depicting the influence of HIV on incidence rates, 1990-2008.⁽³⁾

Based on data extracted from the World Health Organisation's "Global tuberculosis control – a short update to the 2009 report".⁽²⁹⁾ [Reprinted from The Lancet; Vol. number 378; Lawn SD, Zumla AI; Tuberculosis; Pages 57-72; Copyright 2011, with permission from Elsevier.]

2. MICROBIOLOGY

M. tuberculosis is an obligate human pathogen and the causative agent of TB. The bacteria are thin (0.2 to 0.4×2 to $10 \mu\text{m}$), rod-shaped, non-motile and belong to the *Mycobacteriaceae* family, Actinomycetales order, *Actinomycetes* class.⁽³⁰⁾ These bacteria have an unusual cell wall structure that contains a high lipid content as well as *N*-glycolylmuramic acid instead of *N*-acetylmuramic acid [Figure 2].^(30, 31)

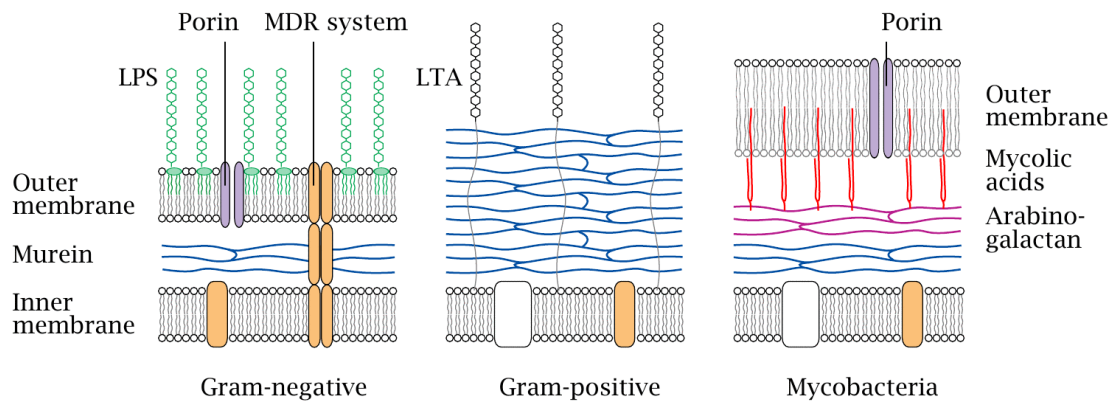


Figure 2. Cell wall structure of mycobacteria compared to gram-negative and gram-positive bacteria.⁽³²⁾

Key: LPS-lipopolysaccharide; LTA-lipoteichoic acid; MDR system- multidrug resistance system.

Because of this unique cell wall structure, mycobacteria are difficult to stain with commonly used staining dyes, such as those used in the Gram stain. These organisms resist decolourisation by acidified alcohol after prolonged application of a basic fuchsin dye or with heating of this dye following its application.⁽³⁰⁾ This important property of mycobacteria that is dependent on its cell wall is referred to as acid-fastness.⁽³⁰⁾ This acid-fast characteristic (although not specific to mycobacteria) distinguishes mycobacteria from other genera.⁽³⁰⁾

Another important feature of these bacteria, in particular *M. tuberculosis*, is that they grow more slowly than most other human pathogenic bacteria, in part because of their hydrophobic cell surface.⁽³⁰⁾ Because of this hydrophobicity, organisms tend to clump, so that nutrients are not easily allowed into the cell.⁽³⁰⁾ Growth is slow or very slow [a generation time of 792-932 minutes for *M. tuberculosis*⁽³³⁾ versus 15-20 minutes for *Escherichia coli*⁽³³⁾], with colonies of mycobacteria becoming visible in two to 60 days at optimum temperature.⁽³⁰⁾

Currently, there are more than 100 recognised or proposed species in the genus *Mycobacterium*.⁽³⁴⁾ These species produce a spectrum of infections in humans and animals ranging from localised lesions to disseminated disease.⁽³⁰⁾ Many species are also environmental organisms and can be found in water and soil.⁽³⁰⁾ For the most part, mycobacteria can be divided into three major groups based on fundamental differences in epidemiology and association with disease: those belonging to the *M. tuberculosis* complex (*M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium pinnipedii*, *Mycobacterium microti*, *Mycobacterium caprae*, and *Mycobacterium canettii*), *Mycobacterium leprae* and *Mycobacterium lepromatosis* (the causative agents of leprosy)⁽³⁵⁾ and those referred to as nontuberculous mycobacteria (NTM).⁽³⁰⁾

The main aetiology of human TB is *M. tuberculosis*. *M. bovis* has a wider host range and is the principal cause of TB in other animal species.^(36, 37) Humans can become infected by *M. bovis*, usually via consumption of unpasteurised milk or milk products from an infected animal⁽³⁸⁾; or disseminated infection may follow administration of the TB vaccine Bacille Calmette-Guerin (BCG) [which contains live attenuated *M. bovis*] in immunocompromised individuals.⁽³⁹⁻⁴³⁾ Other members of the *M. tuberculosis* complex include the human pathogens *M. canettii*^(30, 44-46), *M. africanum*⁽⁴⁷⁾, and several species associated with particular animals such as voles (*M. microti*)⁽⁴⁸⁾, goats and deer (*M. caprae*)⁽⁴⁹⁾, and seals (*M. pinnipedii*).⁽⁵⁰⁾

3. PATHOGENESIS

Mycobacterium tuberculosis is transmitted via “minute aerosol droplets that remain suspended in the air for prolonged periods of time”.⁽⁵¹⁾ Different

outcomes may result following inhalation of an infectious droplet containing the organism.⁽⁵²⁾ The majority of immunocompetent individuals either eliminate *M. tuberculosis* or contain it in a latent state in which an equilibrium is established between the host and pathogen.⁽⁵¹⁾ Latent TB can affect any organ, but the life cycle of *M. tuberculosis* is only completed when it causes pulmonary immunopathology, which drives aerosolisation of bacilli and transmission to a new host.⁽⁵³⁾ The probability of development of active clinical TB after being infected with *M. tuberculosis* is very small. "Less than 10% of those infected develop symptoms and signs of active disease over a lifetime; the actual figure depends on geographical location, *M. tuberculosis* strain type, genetic background, immunosuppression, and other risk factors."⁽⁵¹⁾

In the majority of infected individuals, the initial primary infection is self-limiting. Ninety per cent of those exposed to *M. tuberculosis* are estimated to achieve lifelong control of the infection. In about five per cent of individuals, the infection progresses from a latent form to active disease within two years after infection, and an additional five per cent have active disease at some later point in their lives.^(54, 55) Although the majority of cases of active TB are traditionally believed to arise from a reactivation of latent infection, exogenous reinfection with a second strain of *M. tuberculosis* does occur, particularly in profoundly immunocompromised persons and in those heavily exposed to new bacilli such as in high incidence settings.⁽⁵⁶⁻⁵⁸⁾ In a recent review, Cardona⁽⁵⁸⁾ has concluded that exogenous reinfection together with primary infection appear to be the major sources of adult TB, contrary to what is usually cited in TB reference books.

The TB life cycle (Figure 3) involves an initial seeding event in the well ventilated lung bases, followed by dissemination to a second location at the lung apices, where immunopathology can lead to lung destruction and

transmission via coughing.^(59, 60) Cavitation does not occur in advanced HIV infection, where the inflammatory innate response is weakened, but can develop during immune reconstitution after antiretroviral therapy (ART).⁽⁶¹⁾

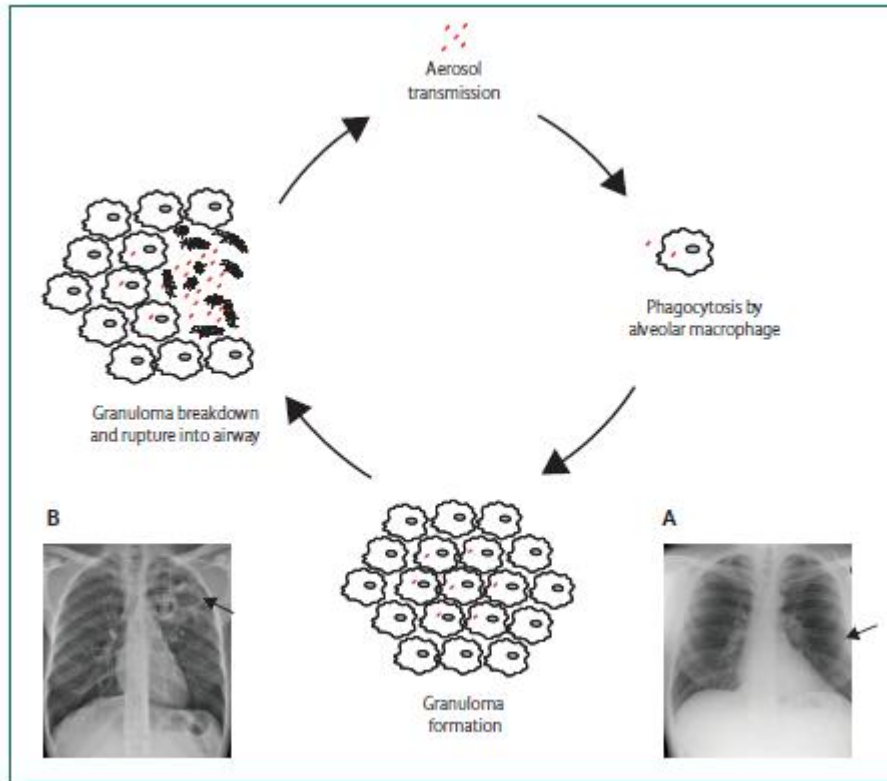


Figure 3. The *M. tuberculosis* life cycle.⁽⁵⁵⁾ “Aerosolised bacilli (red) are inhaled and phagocytosed by alveolar macrophages, which form granuloma that ultimately break down and rupture necrotic lung tissue, thereby releasing bacilli into the airway to reinitiate the infectious cycle.”⁽⁵⁹⁾ [Reprinted from The Lancet; Vol. number 15; Elkington PT, Friedland JS; Permutations of time and place in tuberculosis; Pages 1357-1360; Copyright 2015, with permission from Elsevier.]

Key: A= arrow indicates calcified Ghon focus; B= arrow indicates cavity.

HIV infection has a critical impact on the host’s cell-mediated response to *M. tuberculosis*.⁽⁶²⁾ The risk of TB increases two to threefold within the first two years of HIV seroconversion⁽⁶³⁾ and continues to increase as CD4 T-cell counts decrease.^(64, 65) HIV-infected individuals with *M. tuberculosis* infection have a mean annual risk of developing active TB of approximately 10% per

year; however, “this risk is highly dependent on the degree of immunodeficiency, the prevailing socioeconomic conditions, and ongoing risk of TB exposure.”⁽⁶⁶⁾ People living with HIV (PLWH) are 20 to 37 times more likely to develop TB than those without HIV.^(67, 68) These patients have a higher mortality rate, probably due to profound immunosuppression as well as delayed diagnosis.⁽⁶⁹⁾ Furthermore, HIV-infected individuals often do not manifest typical symptoms of TB such as prolonged cough, fever, night sweats, and weight loss⁽⁷⁰⁾ which makes clinical diagnosis difficult. HIV-infected patients also are more likely to have extrapulmonary TB (EPTB) than HIV-uninfected patients.⁽⁷¹⁾ HIV changes the presentation of smear-negative pulmonary TB (PTB) from a slowly progressive disease with low bacterial load and reasonable prognosis, to one with reduced pulmonary cavity formation and sputum bacillary load^(72, 73), more frequent involvement of the lower lobes^(72, 73), and an exceptionally high mortality rate.⁽⁷⁴⁾ In one study in sub-Saharan Africa, a third of patients with smear-negative TB died within a year of their initial diagnosis.⁽⁷⁵⁾

The two clinical manifestations of active TB are PTB and EPTB. The former is most common (approximately 85%).⁽⁷⁶⁾ EPTB refers to TB involving organs other than the lungs (e.g., pleura, lymph nodes, abdomen, genitourinary tract, skin, joints and bones, or meninges).⁽⁷⁷⁾ The likelihood of EPTB increases as the CD4 T-cell count decreases.⁽⁷⁸⁾ The diagnosis of EPTB poses a particular challenge for clinicians because of the protean ways in which the disease presents. ⁽⁷⁹⁾

In children, the diagnosis of PTB is especially difficult because the disease is paucibacillary and collection of sufficient sputum for smear microscopy and culture is difficult.⁽⁸⁰⁾ As a result of difficulties in paediatric (and to a lesser extent adult) diagnosis, TB treatment is often administered empirically, which leads to underdiagnosis or, in some cases, to overdiagnosis and

subsequent inappropriate prescription of antibiotics to patients without infection.⁽⁸¹⁾ Underdiagnosis leads to increased morbidity and mortality due to TB. Overdiagnosis results in higher treatment costs to TB programmes, patient exposures to unnecessary drug side-effects, and delay in making the correct diagnosis.

4. EPIDEMIOLOGY

In 2013, just over six million cases of TB were reported by 202 countries and territories to the World Health Organisation (WHO) of which 5.7 million cases were newly diagnosed.⁽²⁶⁾ Based on WHO estimates* for 2015 global targets, of the estimated nine million people who developed TB in 2013, more than half (56%) were in the South-East Asia and Western Pacific Regions [Figure 4].⁽²⁶⁾ A further one quarter were in the African Region, which also had the highest rates of cases and deaths relative to the population [Figure 5].⁽²⁶⁾ The HIV epidemic in sub-Saharan Africa has increased the caseload of TB. An estimated 1.1 million (13%) of the nine million people who developed TB in 2013 were HIV-infected.⁽²⁶⁾ The African Region accounts for about four out of every five HIV-infected TB cases and TB deaths among people who were HIV-infected.⁽²⁶⁾ Tuberculosis kills one individual every 18 seconds and infects an individual every second.⁽⁸²⁾ In 2009, there were almost 10 million children who were orphaned as a result of parental deaths caused by TB.⁽⁸³⁾ These statistics illustrate the huge consequences TB has not only on an individual level but on a societal level as well.

* Because no countries undertake nationwide surveys of TB incidence, WHO estimates are based on estimates of the cases detected, data on case notifications and expert opinion (high-income countries), and empirical measures of disease prevalence.⁽²⁶⁾ A notification is a case where TB is diagnosed in a patient and is reported within the national surveillance system, and then on to WHO.⁽²⁶⁾ Therefore, estimates will be higher than case notification rates (which depend on accuracy of the country notification systems). In 2013, survey results for five new countries were

finalised: Gambia, Lao PDR, Nigeria, Pakistan and Rwanda.⁽²⁶⁾ When new data becomes available, this may affect the global TB estimates. Given the size of the Nigerian population and the high TB burden in Nigeria, the WHO had to revise its 2014 estimates upwards.⁽²⁶⁾

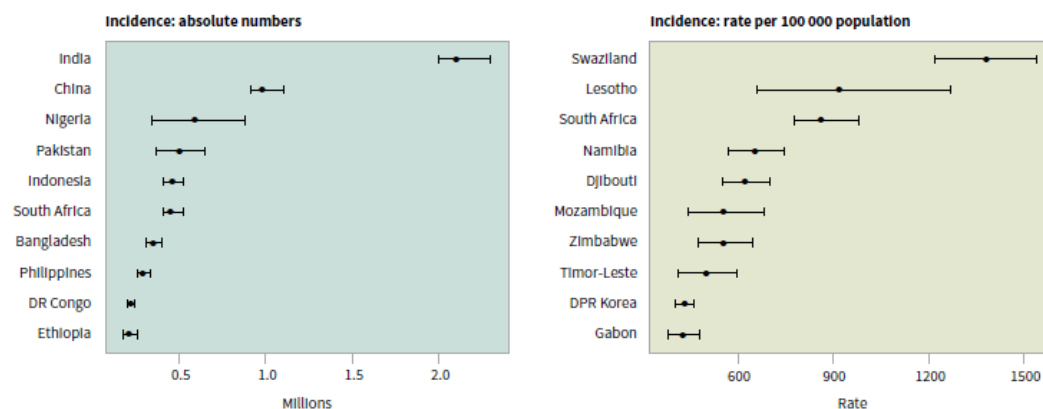


Figure 4. World Health Organisation's estimated TB incidence of the top ten countries in 2013.

Reproduced from the World Health Organisation's "Global Tuberculosis Report 2014".⁽²⁶⁾

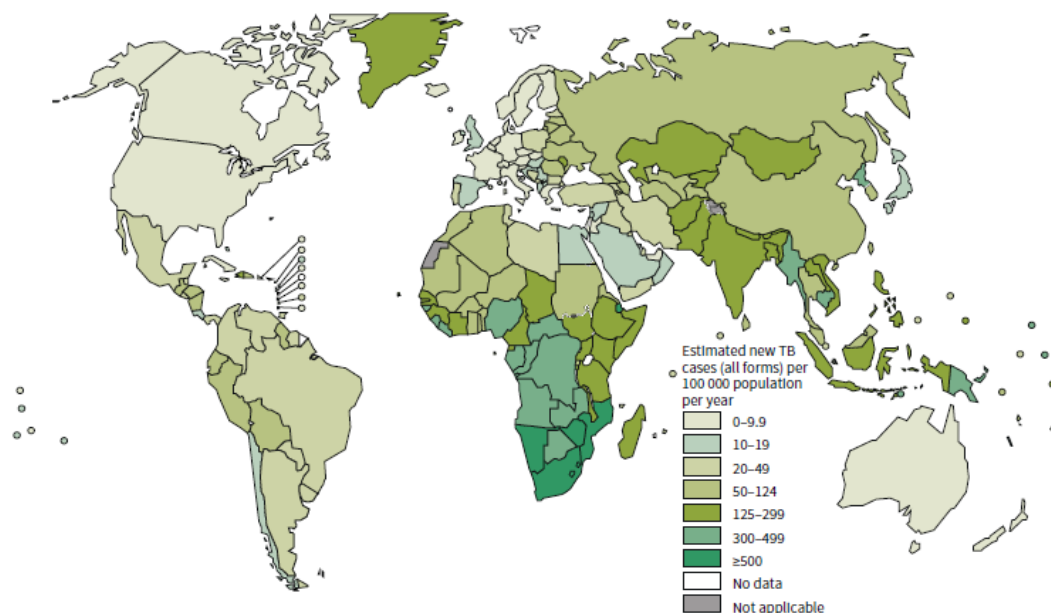


Figure 5. World Health Organisation's estimated TB incidence rates, 2013.

Reproduced from the World Health Organisation's "Global Tuberculosis Report 2014".⁽²⁶⁾

Multidrug-resistant (MDR) TB [defined as resistance to at least rifampicin (RIF) and isoniazid (INH), two of the most powerful antituberculosis drugs] is now a major health problem.⁽²⁶⁾ Globally, an estimated 3.5% (95% confidence interval (CI) [2.2, 4.7]) of new cases and 20.5% (95% CI [13.6, 27.5]) of previously treated cases have MDR-TB.⁽²⁶⁾ In 2013, there were an estimated 480,000 (range: 350,000-610,000) new cases of MDR-TB worldwide, and approximately 210,000 (range: 130,000-290,000) deaths from MDR-TB.⁽²⁶⁾ Globally, only 8.5% of new bacteriologically confirmed TB cases and 17% of those previously treated for TB were tested for drug resistance in 2013.⁽²⁶⁾ In 2008, 39% of all estimated new cases and 97% of the incident cases of MDR-TB were not detected by national TB programmes.⁽²⁹⁾ Failure to detect drug resistance results in inappropriate treatment and possibly premature death of the individual patient, but it also facilitates amplification of resistance and ongoing transmission within the community, greatly worsening the situation.⁽⁸⁴⁾

One of the most important factors influencing the current TB epidemic in resource-limited settings is poverty, which is closely related to malnutrition⁽⁸⁵⁾, crowded living conditions and lack of access to free or affordable health care services.⁽²⁸⁾ The slow onset and lack of specific symptoms makes the disease difficult to recognise in the early stages and patients may delay for weeks or months before seeking medical assistance, during which time they may transmit the disease to others.^(86, 87) Recent estimates of patient delays in seeking care, particularly among HIV-negative TB patients, suggest a duration of smear positivity before diagnosis of >one year in Africa and even longer in many parts of Asia.⁽⁸⁸⁾ When patients eventually seek care at their local health centre, access to treatment may be delayed due to the lack of effective diagnostic tools, with the detection of early-stage disease, EPTB, HIV co-infected, and paediatric cases being particularly difficult.⁽⁸⁹⁾

Despite efforts to increase case detection, an estimated third of TB cases are still being missed each year.⁽⁹⁰⁾ Lonnroth *et al.*⁽²⁷⁾ calculated in 2009 that if we were to eliminate TB as a major health problem by 2050 (< 1 case per 1 million population per year), we would need to reduce the incidence by an average of 16% annually over the next 40 years. Targets for the WHO post-2015 global TB strategy include a 95% reduction in TB deaths and a 90% reduction in TB incidence by 2035.⁽⁹¹⁾

The diagnosis of TB remains clinically challenging and logistically difficult in resource-limited settings.^(92, 93) In Africa and South-East Asia (the WHO regions most heavily affected by TB), per capita government expenditure on health during 2007 was just US \$34 and \$15, respectively.⁽⁹⁴⁾ The lack of laboratory facilities makes the laboratory diagnosis of infectious diseases difficult in many parts of the African continent.^(28, 93) The vast majority of patients suspected of having TB in endemic countries present to peripheral healthcare facilities that may have no electricity, no running water, and limited or no laboratory facilities.^(51, 93) Denkinger *et al.*⁽⁹⁵⁾ who performed a survey of the conditions of peripheral microscopy centres of the 22 high-burden countries found that only four out of the 22 high-burden countries (18%) reported access to uninterrupted power supply, while a supply of running water was available in 13 (59%) of these countries. Microscopy centres in six countries had refrigerators (27%), and only in five (23%) countries was a centrifuge consistently available.⁽⁹⁵⁾

5. DIAGNOSIS OF TB

While upstream factors such as alleviation of poverty, improvement in housing and transport, and reduction in the rates of HIV co-infection, and downstream factors such as availability of effective TB drugs, adherence and

active case finding are clearly important, the implementation of new diagnostics is critical.⁽⁷⁶⁾ Whilst rapid and accurate diagnosis represents only one facet of TB control, it is a fundamental one.⁽⁷⁶⁾ Early diagnosis of TB and universal drug susceptibility testing (DST) are the first steps necessary to identify adequate treatment for individual patients and to prevent the spread of disease at the population level.⁽⁹⁶⁾ It is estimated that availability of a widely used rapid diagnostic test for TB that was 100% accurate and that led to initiation of treatment could avert 625,000 TB deaths each year.⁽⁹⁷⁾

5.1 Limitations and challenges of existing diagnostics used for the diagnosis of active TB

Despite the long existence of TB infections in humans, diagnostic techniques in many developing countries are similar to those used in the 1800's. Of the 5.2 million incident PTB patients notified globally in 2014, only three million (58%) were bacteriologically confirmed, i.e., were smear- or culture-positive or positive using a WHO-recommended rapid diagnostic such as Xpert®MTB/RIF assay (Cepheid, Sunnyvale, USA).⁽²⁶⁾ In high incidence countries, TB control often relies on passive case-finding among individuals self-presenting to healthcare facilities, followed by either diagnosis based on clinical symptoms or laboratory diagnosis using sputum smear microscopy.⁽²⁸⁾ Serial sputum specimens are required which means that the patients are asked to make repeated visits to the healthcare centre for specimen delivery (if they are unable to expectorate two spot specimens on their initial visit to the clinic in countries which have adopted a frontloaded ['same day' or 'one-stop'] approach) and for subsequent collection of their microscopy results.^(28, 98) For many patients, the costs of repeated visits to healthcare facilities are prohibitive, and patient dropout is a significant problem.⁽²⁸⁾ Given the high mortality in the HIV/TB co-infected population,

the need to detect TB accurately and rapidly is great, yet the performance of most tests is diminished in this group of patients.

This remainder of this review will focus on current diagnostic tools and tests for TB, particularly those in use in low-income settings. Only diagnosis of active TB (not latent infection) will be discussed in depth. Diagnostic techniques are summarised in Figure 6.

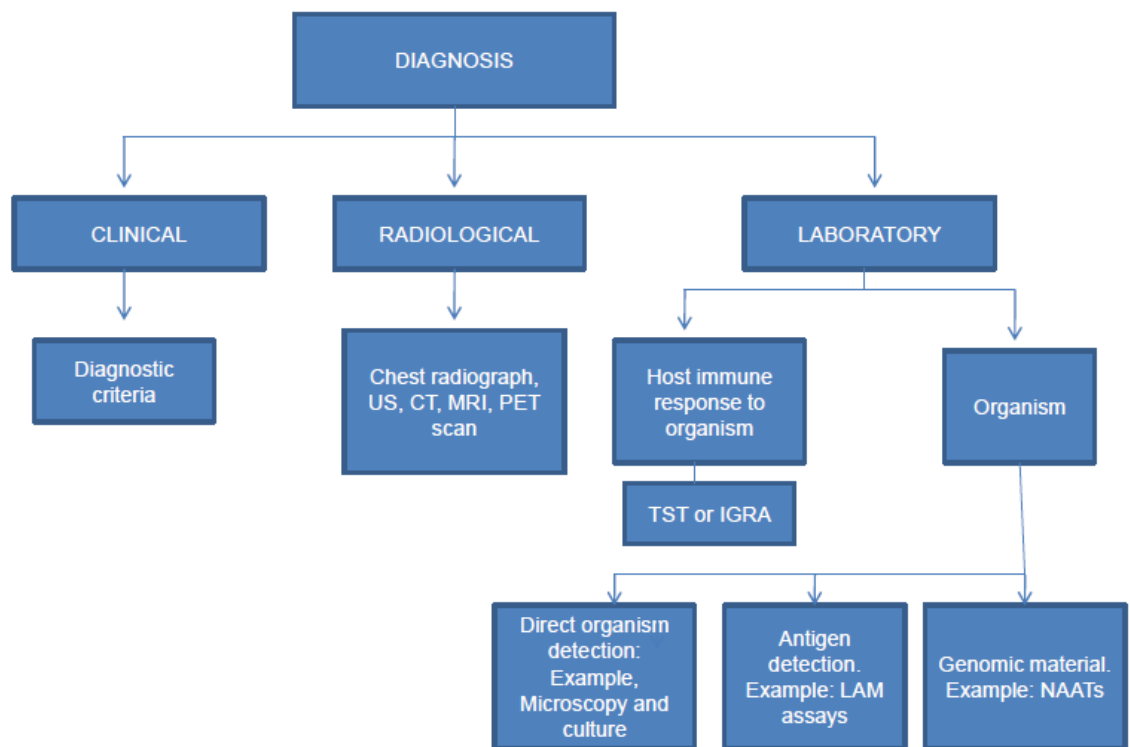


Figure 6. Current conventional approach to diagnosis of active TB.

Key: US-ultrasound; CT-computerised tomography; MRI-magnetic resonance imaging; PET-positron emission tomography; TST-tuberculin skin test; IGRA-interferon gamma release assay; LAM-lipoarabinomannan; NAATs-nucleic acid amplification tests.

5.1.1 Screening using clinical symptoms and signs (clinical diagnostic criteria)

Due to problems with existing laboratory diagnostics, clinical algorithms for syndromic management have been developed and evaluated. These clinical scoring systems often combine some non-invasive tests (e.g., radiography) in combination with clinical symptoms and/or signs. Clinical symptoms and signs of TB include cough, fever, night sweats and weight loss. These symptoms and signs are not specific to TB and can be seen in other conditions.⁽⁹⁹⁻¹⁰¹⁾ A postmortem study of 93 South African HIV-infected children who presented with respiratory difficulty found that the clinical presentation of PTB was virtually indistinguishable from respiratory failure caused by *Pneumocystis jiroveci*, cytomegalovirus, bacterial pneumonia, or lymphocytic interstitial pneumonitis.⁽¹⁰²⁾ Screening tools that combine multiple symptoms have much higher sensitivity, albeit with low specificity.⁽¹⁰³⁻¹⁰⁵⁾ Van Hoogt *et al.*⁽⁹⁹⁾ reported that if prolonged cough was present (which in itself has low sensitivity) as either the only screen or a first step followed by chest radiography (CXR), the sensitivity of the clinical screen is low: 22% if the reference test for screen-positives is sputum smear microscopy and approximately 30% if Xpert®MTB/RIF assay is used as the reference.

Clinical scoring systems have probably been best studied in the paediatric population due to the difficulty of making the diagnosis of TB in this population. Frigati *et al.*⁽¹⁰⁶⁾ found that prolonged fever (>1 week), CXR suggestive of TB or a positive tuberculin skin test (TST) were predictive of definite TB and should be considered in composite scoring systems for TB diagnosis in the paediatric population in high HIV prevalence settings. Edwards *et al.*⁽¹⁰⁷⁾ who retrospectively evaluated eight different diagnostic scoring systems used to diagnose TB among 91 children at a paediatric hospital in Kinshasa, Democratic Republic of Congo, over a one year period,

found that correlation between the different scoring systems was poor and there was considerable disagreement on when to initiate TB treatment.

Scoring systems are generally not well validated and validation is hampered by the lack of a suitable reference standard for comparison particularly in the paediatric or HIV-infected population.⁽¹⁰⁸⁾ There is also marked variation in performance between these diagnostic approaches.^(108, 109) In addition, there are several limitations in many of the studies conducted to validate such scoring systems, such as the use of appropriate case definitions, choice of reference testing, selection bias, small numbers, methodological differences and heterogeneity of the study population.^(108, 110) Generally these scoring systems perform less well in important sub-groups that pose the greatest diagnostic challenge and are at greatest risk for poor outcome, such as the young, malnourished or HIV-infected.^(100, 108, 109, 111)

The World Health Organisation TB symptom screening algorithm (WHO-TB) has been recommended for TB screening in PLHIV.⁽¹¹⁰⁾ This screening tool is based on the self-reported presence of any of four symptoms found to be associated with active TB in PLHIV (weight loss, fever, night sweats, and cough of any duration).⁽¹¹⁰⁾ The WHO-TB screen is based on pooled point estimates of sensitivity and specificity (Table 1). Because the WHO-TB has a high negative predictive value (proportion of cases with a negative screen that truly don't have TB), it is mainly used as a "rule-out" test for TB.⁽¹¹⁰⁾ However, the positive predictive value (proportion of cases screening positive that truly have TB) of the WHO-TB for detection of TB is low.⁽¹¹⁰⁾ In patients with HIV, the presence of cough of any duration, fever of any duration, or night sweats lasting three or more weeks in the preceding four weeks was found to be 93% sensitive and 36% specific for TB in a high incidence region in a recently published analysis.⁽¹¹²⁾ Because the proportion of PLHIV reporting WHO-TB symptoms can be as high as 80%, many PLHIV

will require further TB investigations prior to initiation of ART and isoniazid preventative therapy (IPT).⁽¹¹³⁾ In a meta-analysis of 8,148 PLHIV screened, prolonged cough was reported by 1,530 (20%) of participants, whilst one or more of the four WHO symptoms was reported by 3,563 (46.6%) of participants.⁽¹¹⁴⁾ Modi *et al.*⁽¹¹⁵⁾ in a prospective study investigating the performance of the WHO algorithm for TB screening in 1,157 Kenyan PLHIV reported a sensitivity and specificity of 74.4% and 49.4%, respectively. These authors found that if TB diagnostic testing were limited to PLHIV reporting symptoms in the WHO algorithm, 25% of PLHIV with laboratory-confirmed PTB would have been missed.⁽¹¹⁵⁾ As the WHO-TB has significant shortcomings, especially for HIV-infected persons in ART programmes in which the prevalence of TB is high, there is a strong argument for microbiological screening of all patients regardless of the presence or absence of symptoms.^(113, 116, 117)

Table 1. Pooled sensitivity and specificity of different screening tools for pulmonary TB using culture-confirmed pulmonary TB as the reference standard.

Reproduced from the World Health Organisation's "Systematic screening for active tuberculosis. Principles and recommendations."⁽¹¹⁰⁾

Screening tool	Pooled sensitivity % (95% confidence interval)	Pooled specificity % (95% confidence interval)
<i>Chest radiography</i>		
Any abnormality compatible with TB (active or inactive)	98 (95–100)	75 (72–79)
Abnormalities suggestive of active TB	87 (79–95)	89 (87–92)
After positive screening for symptoms ^a	90 (81–96)	56 (54–58)
<i>Symptom screening</i>		
Prolonged cough (lasting >2–3 weeks)	35 (24–46)	95 (93–97)
Any cough	57 (40–74)	80 (69–90)
Any TB symptom (settings with low prevalence of HIV)	70 (58–82)	61 (35–87)
Any TB symptom (settings with high prevalence of HIV)	84 (76–93)	74 (53–95)
Any TB symptom (settings with low prevalence or high prevalence of HIV)	77 (68–86)	68 (50–85)

Key: ^a Results from only one study, data for any abnormality on chest radiography."⁽¹¹⁰⁾

In summary, the better validated systems may have a role as a screening tool in some settings, but have poor specificity and are unlikely to replace other diagnostic modalities.

5.1.2 Radiology

Chest radiography is a sensitive but non-specific test to detect TB.^(100, 118, 119) It cannot be used solely to establish a diagnosis of TB as radiographs may be normal or show non-specific changes in patients with active TB.⁽¹²⁰⁻¹²²⁾ The radiographic diagnosis of TB is initially correct in only 49% of all cases-34% for the diagnosis of primary TB, and 59% for the diagnosis of reactivation TB.⁽¹²¹⁾ In the immunocompetent adult, upper lobe cavitary disease is typical (Figure 7), whereas lower lung zone disease, adenopathy, and effusions, which are uncommon in adults, are the hallmarks of TB in an immunocompromised patient (Figure 8).⁽¹²³⁾ In reality these interpretative markers are not as simple as there is significant overlap in radiological presentation. Cavitation, the radiological hallmark of PTB, is radiographically evident in only 20–45% of patients.^(124, 125)

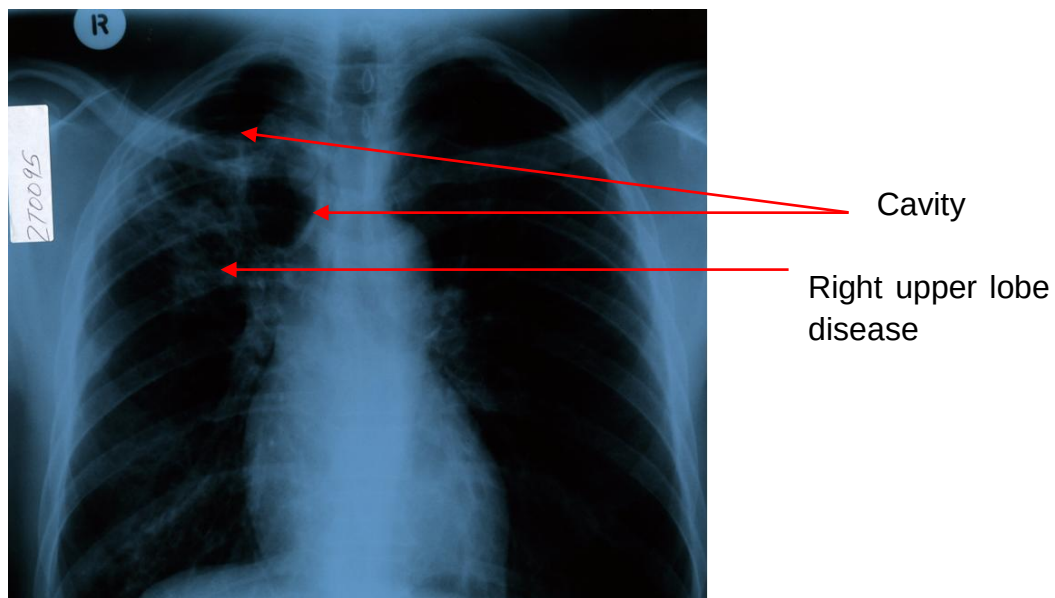


Figure 7. Typical radiographic findings of TB with right upper lobe disease and cavity formation.

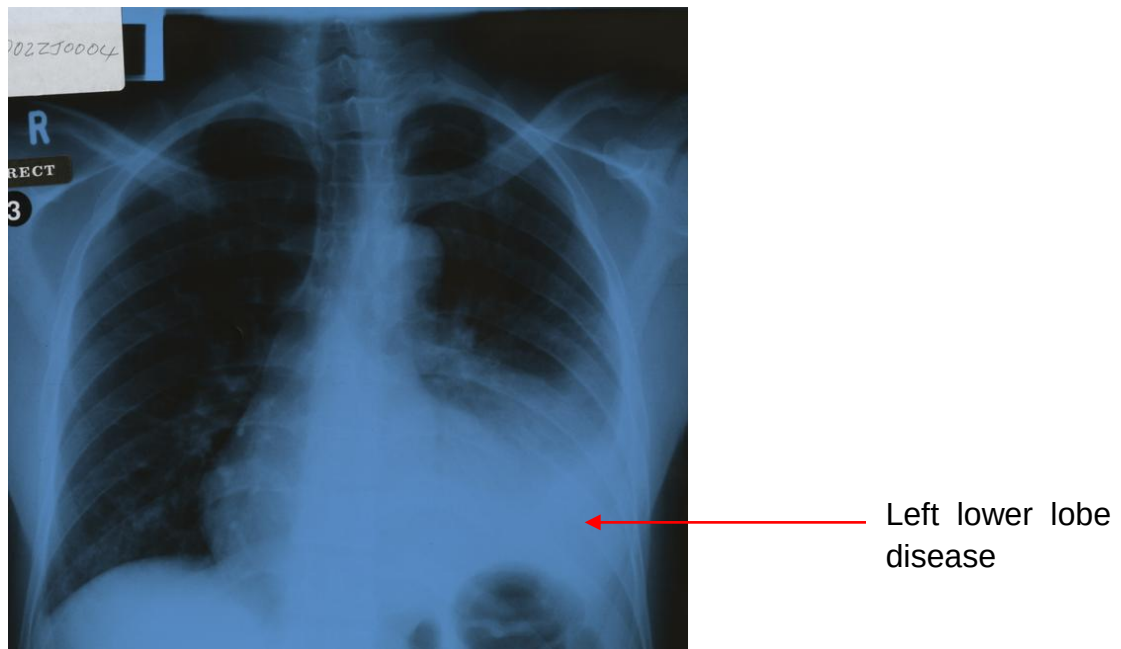


Figure 8. Chest radiograph showing left lower lobe opacification. Note lack of cavitation in this patient with a low CD4 T-cell count.

HIV infection further diminishes the reliability of CXR in the diagnosis of PTB, since the disease commonly presents with an atypical pattern.^(68, 100, 126) In areas of high HIV and TB prevalence, 75% of patients with smear-negative TB are likely to have atypical CXR findings.⁽¹²⁷⁾ Such patients characteristically demonstrate unusual patterns, for example middle and lower lung involvement, absence of cavity formation, presence of lymphadenopathy, pleural effusions, or a miliary pattern.^(124, 128) Getahun *et al.*⁽⁷²⁾ found that the CXR may be normal in up to 14% of HIV-infected patients with sputum culture-positive TB. In a study involving patients accessing ART in Cape Town, South Africa, the sensitivity of any radiographic abnormalities consistent with TB was just 68% and the specificity was 53%.⁽¹²⁹⁾ Thus, one-third of patients found to have sputum culture-positive TB by active screening had completely normal CXR findings.⁽¹²⁹⁾

The radiographic appearance of HIV-associated PTB has been found to be dependent on the level of immunosuppression at the time of overt disease.⁽¹³⁰⁾ Radiological manifestations in patients with a CD4 T-lymphocyte count of <200 cells/mm³ show a higher incidence of mediastinal or hilar lymph node enlargement, a lower prevalence of cavitation, and often extrapulmonary involvement compared with HIV-infected patients with a CD4 T-lymphocyte count of ≥ 200 cells/mm³.⁽¹³⁰⁾

Additional problems associated with the use of CXR for the diagnosis of PTB are poor film quality and difficulties with interpretation.⁽¹³¹⁻¹³³⁾ A survey in Malawi showed that medical officers misdiagnosed a third of clinical vignettes, which described typical radiographic signs of TB.⁽¹³⁴⁾ Radiology machines are not available at the majority of healthcare facilities in low- and middle-income settings, particularly at microscopy or primary health centres.

In primary PTB, CXR remains the mainstay for the diagnosis of parenchymal disease, while computed tomography (CT) is more sensitive in detecting lymphadenopathy.⁽¹²⁴⁾ In post-primary PTB, CT is the method of choice to reveal early bronchogenic spread.⁽¹²⁴⁾ In a study of 41 patients with active TB, high-resolution CT showed cavities in 58%, whereas CXR showed cavities in only 22%.⁽¹³⁵⁾ Newer tests such as magnetic resonance imaging (MRI) and ¹⁸F-fluorodeoxyglucose positron emission tomography/ computerised tomography (¹⁸F-FDG PET/CT) may have a role in TB diagnosis.^(122, 124) Fluorodeoxyglucose (FDG) accumulates in metabolically active immune cells and can serve as a marker of TB-associated inflammation.⁽¹³⁶⁾ ¹⁸F-FDG PET-CT at two months has been shown in pilot studies to correlate with outcome in patients with confirmed MDR-TB.⁽¹³⁷⁾ However, as FDG accumulates in all metabolically active cells, it has low specificity as a diagnostic test and is unable to distinguish between TB and other metabolically active lung

processes such as malignancy.⁽¹³⁶⁾ In addition, due to the high cost, these technologies remain out of reach of low- and middle-income countries.

The introduction of digital radiography has improved image quality and facilitates the storage and sharing of images and, if required, a second opinion may be sought by remote (electronic) access.⁽¹³⁶⁾ Compared to film-based radiographs, running costs are reduced and reagent stock-outs avoided, but the cost of buying/leasing and maintaining the equipment remains high.⁽¹³⁶⁾ Application of computer-aided image analysis to provide an automated imaging service is being researched. Studies suggest that sensitivities from automated readers can be similar to those obtained by eye but that specificity is reduced; a finding confirmed by studies in Africa.⁽¹³⁸⁻¹⁴⁰⁾

5.1.3 Serology

There are currently over “40 rapid serologic TB tests (that use various antigenic compositions to detect patients’ antibodies) available in many low- and middle-income countries.”⁽²⁸⁾ These tests differ in a number of features, including antigen composition, antigen source (e.g., native or recombinant), chemical composition (e.g., protein, carbohydrate, or lipid), extent and manner of purification of the antigen(s), and class of immunoglobulin detected (e.g., IgG, IgM, or IgA).⁽²⁸⁾ Systematic reviews^(141, 142) have reported strong evidence that existing commercial serologic tests are of little clinical value because of suboptimal accuracy and highly inconsistent results. In a comparative study of 19 different tests, the highest sensitivity observed was 59.7%, with some tests detecting less than one in 10 TB patients.⁽¹⁴²⁾

Kunnath-Velayudhan *et al.*⁽¹⁴³⁾ have developed microarrays of over 4,000 *M. tuberculosis* proteins and probed those arrays with serum from individuals

whose TB status had been characterised. This approach has identified the *M. tuberculosis* ‘immunoproteome’, i.e., the set of *M. tuberculosis* proteins that are recognised by the humoral immune system.⁽¹⁴³⁾ Work to identify sets of antibody responses that are associated with TB disease states is underway, and holds some promise for development of a serology-based diagnostic test.⁽¹⁴⁴⁾

5.1.4 Tuberculin skin test and interferon gamma release assays

The TST is based on a delayed-type hypersensitivity reaction that occurs when those infected with *M. tuberculosis* are exposed to certain antigenic components present in extracts of culture filtrates, the “tuberculin.”⁽¹⁴⁵⁾ In this type of reaction, T cells, sensitised by prior infection, are recruited to the skin where the tuberculin was injected and release lymphokines.⁽¹⁴⁵⁾ The result is local induration of the skin through local vasodilatation, oedema, fibrin deposition, and recruitment of other inflammatory cells to the area.⁽¹⁴⁵⁾ The test has been used to identify those who may benefit from IPT and aid diagnosis of TB disease, especially in children. The sensitivity of TSTs for detecting TB infection is impaired among PLHIV due to an attenuation of cell-mediated responses, including a reduction in memory CD4 T-cells at the TST site.⁽¹⁴⁶⁾ While a cut-off of ≥ 10 mm of induration is often used to define a positive TST among those who are HIV sero-negative, a cut-off of ≥ 5 mm is instead recommended among PLWH to increase the sensitivity.^(147, 148) A systematic review by Farhat *et al.*⁽¹⁴⁹⁾ found that the effect on TST of BCG received in infancy is minimal, and virtually nil 10 years or more after vaccination. However, BCG received after infancy produces more frequent, more persistent and larger TST reactions.⁽¹⁴⁹⁾

The interferon gamma release assay (IGRA), a blood test measuring cellular immune response to *M. tuberculosis* antigens, assesses infection in a manner similar to the traditional TST, but with higher specificity than TST. However, like TST, IGRAs are limited by their inability to differentiate between infection and disease and are dependent on host-responses, which may be compromised in immunocompromised patients.^(150, 151) Recent reviews and meta-analyses concluded that neither IGRAs nor the TST have high accuracy for the prediction of active TB, although use of IGRAs in certain populations might reduce the number of people being considered for preventive treatment.⁽¹⁵¹⁻¹⁵³⁾ A systematic review and meta-analysis⁽¹⁵⁴⁾ in the paediatric population showed that in microbiologically confirmed cases, TST sensitivity was 86% (95% CI [79, 91]) in high-income countries, and 74% (95% CI [68, 80]) in low-income countries. A higher IGRA specificity with respect to TST was observed in high-income countries (specificity of 97-98% for IGRA vs. 92% for TST), but this increased specificity of IGRA compared to TST was not observed in low-income countries (85-93% vs. 90%).⁽¹⁵⁴⁾ The WHO has issued negative recommendations regarding the use of serodiagnostic tests and IGRAs for diagnosis of TB or latent *M tuberculosis* infection in low- and middle-income countries.⁽¹⁵²⁾

5.1.5 Microbiological testing

Since 1882 when Koch first demonstrated the aetiological agent responsible for TB, the diagnosis of TB has relied primarily on the microbiology laboratory.^(101, 155)

5.1.5.1 Sputum smear microscopy

Sputum smear microscopy remains the cornerstone of TB diagnosis in most of the high burden countries.^(28, 156) Kik *et al.*⁽¹⁵¹⁾ reported that in the 22 high burden countries, over 77 million smears are performed annually. In 2011, approximately five million sputum smears were performed in South Africa, reflecting the severity of the South African TB epidemic.⁽¹⁵⁷⁾ Smear microscopy is feasible in most settings and it is highly specific for *M. tuberculosis* in TB-endemic countries.⁽¹⁵⁸⁾ Not only is microscopy rapid, it identifies those patients who are most likely to transmit infection.⁽¹⁵⁹⁾

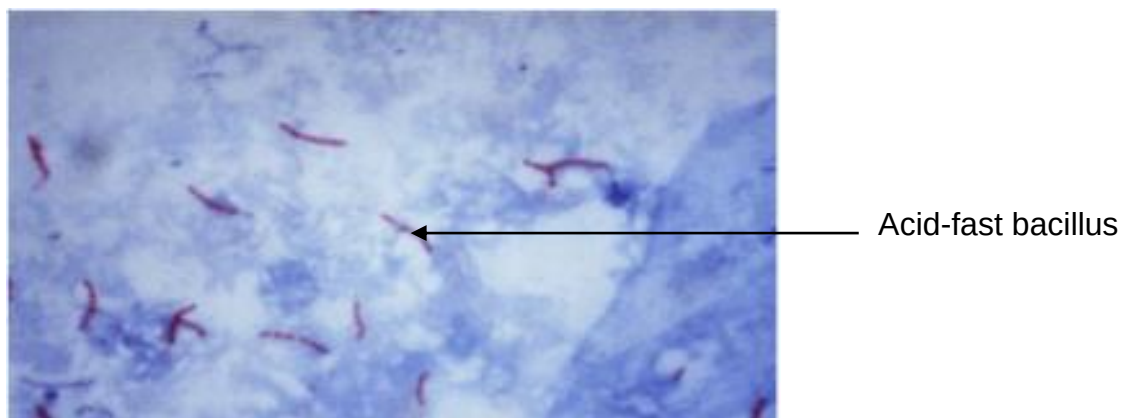


Figure 9. Acid-fast bacilli (Ziehl-Neelsen stain).⁽¹⁶⁰⁾

The current WHO international policy on TB case detection recommends the collection and examination of two sputum smears for the diagnosis of PTB.⁽¹⁶¹⁾ The change from three to two smears is based on a systematic review of 37 studies that quantified the incremental diagnostic yield of serial sputum specimens.⁽¹⁶²⁾ The results indicated that almost 85.8% of TB cases were detected with the first sputum specimen.⁽¹⁶²⁾

The sensitivity of smear microscopy, however, varies between 20-80%, depending on the quality of specimen submitted, site of infection, and diligence with which smears are made and examined.^(28, 158, 163) For a smear to be positive, there must be at least 5,000-10,000 acid-fast bacilli (AFB) per millilitre of sputum⁽¹⁶⁴⁾, but these bacilli could be released only intermittently from cavities.⁽⁷²⁾ In certain population groups, the sensitivity is lower, particularly in HIV-infected and paediatric patients.^(28, 72, 165) In paediatric patients, the sensitivity is approximately 10-15%.^(166, 167)

The sensitivity of smear microscopy is particularly low in HIV-infected patients (around 20%) and this has resulted in increases in smear-negative TB.^(72, 168) HIV-infected individuals have a higher rate of smear-negative disease because they are less likely to have cavitary lesions due to the impairment of granuloma formation.^(126, 169) Getahun *et al.*⁽⁷²⁾ reported that the proportion of cases of smear-negative PTB in HIV-infected TB patients ranged from approximately 24% to 61%. In TB screening studies in South African ART cohorts, more than 80% of culture-proven TB cases were sputum smear-negative.^(113, 117) In addition, smear-negative TB in HIV-infected individuals is associated with excessive early mortality compared to smear-positive disease.⁽⁷²⁾ Kang'ombe *et al.*⁽⁶⁹⁾ reported that patients with smear-negative PTB had a significantly higher risk of death than patients with smear-positive TB, with a hazard ratio of 2.2. A longer health-service delay in the diagnosis of smear-negative than smear-positive PTB has been reported, perhaps because the diagnostic algorithm needs 11-34 days to establish the diagnosis of smear-negative PTB under the most optimistic scenarios.⁽⁷²⁾ Usually patients receive a course of antibiotics with further clinical assessment, specimen collection and radiology (Table 2).

Table 2. National TB control programme recommendations of selected countries for diagnosis of smear-negative PTB.⁽⁷²⁾

	Smear samples: acid-fast bacilli before antibiotic treatment	Courses of antibiotics*	Smear samples: acid-fast bacilli after unsuccessful antibiotic treatment	Chest radiograph after unsuccessful antibiotic treatment	Clinical assessment after successful antibiotic treatment	Estimated time until diagnosis of SNP (days)†
Cambodia, (2003)	3x (1 set) 3 specimens	2 (1–2 weeks)	3x (1 set) 3 specimens	Yes	Yes	20
Côte d'Ivoire, (2003)	3x (1 set) 3 specimens	2 (7–10 days)	3x (1 set) 3 specimens	Yes	Yes	16
Ethiopia, (2002)	3x (1 set), 2x (1 set) 5 specimens	1 (7–10 days)	3x (1 set) 3 specimens	Yes	Yes	18
India (2005)	3x (1 set) 3 specimens	1 (10–14 days)	3x (1 set) 3 specimens	Yes	No	20
Kenya (2003)	3x (1 set) 3 specimens	1 (5–7 days)	3x (1 set) 3 specimens	Yes‡	No	11
Laos, (2004)	3x (1 set) 3 specimens	1 (2 weeks)	3x (1 set) 3 specimens	Yes	No	25
Lesotho (2005)	3x (1 set) 3 specimens	1 (10–14 days)	3x (1 set) 3 specimens	Yes	No	20
Mozambique, (2004)	2x (1 set) 2 specimens	2 (7–15 days)	2x (1 set) 2 specimens	No§	Yes	21
Malawi, (2002)	2x (1 set) 2 specimens	1 (1 week)	None¶	Yes	No	11
Sri Lanka (2005)	3x (1 set) 3 specimens	1 (1–2 weeks)	3x (1 set) 3 specimens	Yes	Yes	20
Sudan, (2000)	3x (1 set) 3 specimens	1 (1 week)	3x (1 set) 3 specimens	Yes	No	13
Swaziland (2004)	3x (1 set) 3 specimens	1 (1 week)	--	--	--	--
Tajikistan, (2003)	3x (1 set) 3 specimens	1 (7–14 days)	3x (1 set) 3 specimens	Yes	Yes	20
Tanzania, (2003)	3x (2 sets) 6 specimens	1 (14 days)	3x (1 set) 3 specimens	Yes	No	22
Uganda, (2002)	3x (1 set) 3 specimens	1 (1 week)	3x (1 set) 3 specimens	Yes	No	13
Zambia, (2001)	3x (1 set) 3 specimens	2 (3–4 weeks)	3x (1 set) 3 specimens	Yes	Yes	34
Zimbabwe, (1999)	3x (1 set) 3 specimens	2	3x (1 set) 3 specimens	Yes	--	--
WHO (2003)	3x (1 set) 3 specimens	1 (..)	3x (1 set) 3 specimens	Yes	Yes	13

Reprinted from the Lancet; Vol. number 369; Getahun H, Harrington M, O'Brien R, Nunn P; Diagnosis of smear-negative pulmonary tuberculosis in people with HIV infection or AIDS in resource-constrained settings: informing urgent policy changes; Pages 2042-2049; Copyright 2007, with permission from Elsevier.

Key: "SNP-smear-negative PTB. Best scenario assumption: two days to obtain one set (2–3 samples) sputum examination result, two days to obtain chest radiograph, two days for

clinical peer review, plus the maximum duration for the antibiotic course specified. Sputum examinations, chest radiograph, and clinical consultations done in the same facility. *Duration of each treatment; †This scenario assumes the activities to be done sequentially, which might not always be the case; ‡Timed with repeat smear samples; § Before antibiotic treatment; ¶ Direct to chest radiograph; || Not specified, but for sufficient period".⁽⁷²⁾

Microscopy to detect AFB can be improved by sputum liquefaction and concentration by centrifugation or gravity sedimentation.^(72, 170, 171) A systematic review⁽¹⁷²⁾ of 83 studies describing the effects of various physical and/or chemical methods for concentrating and processing sputum prior to microscopy found that concentration resulted in a higher sensitivity (15–20% increase) and smear-positivity rate, when compared with direct smears. The main problem with this is that the majority of microscopy centres lack centrifuges and that sodium hypochlorite which is frequently used as a solvent involves longer processing as well as it is mycobactericidal which substantially reduces the yield of mycobacterial culture if the sputum is to be cultured.⁽¹⁷³⁾ Centrifugation is also not feasible in all settings as it requires a stable electrical supply and increases the biosafety risk for lab workers.⁽¹⁷⁴⁾ Sputum processing procedures involving centrifugation should be performed in a biological safety cabinet and, preferably, a refrigerated centrifuge with sealed safety cups and aerosol tight rotors.⁽²⁶⁾ The purchase and maintenance of these instruments may be challenging for laboratories in resource-poor settings.⁽²⁸⁾ Therefore, other simpler processing methods that do not require these instruments should be considered, especially for rural areas with limited funds, large sample volumes, and the potential for contaminated specimens due to the lack of refrigeration following collection or cold chain during transport.⁽²⁸⁾

Alternative concentration techniques have been researched in an attempt to improve existing staining methods. Overnight sedimentation is a technique that may be used in laboratories lacking electrical supply or centrifuges. An

experimental comparative study by Rasheed *et al.*⁽¹⁷⁵⁾ of 660 smears from 220 sputum specimens showed that compared with direct microscopy, positivity increased by 91.1% after centrifugation and by 71.1% after overnight sedimentation. There was no statistically significant difference observed between the two concentration methods ($p>0.05$, $\chi^2=0.79$).⁽¹⁷⁵⁾ The utilisation of magnetic beads to concentrate mycobacteria present within a specimen is a method that can be performed in a biosafety cabinet with just a magnet. Microsens Medtech Ltd (London, UK) has developed a kit with paramagnetic beads coated with a chemical ligand that bind to mycobacteria. Bacterial cells are captured from the sputum of patients and then stained with auramine phenol for fluorescent microscopy (FM). This simple approach could enhance the sensitivity of microscopy by concentrating the sample, without the need for centrifugation.⁽²⁸⁾ A recent study⁽¹⁷⁶⁾ found that microscopy of the TB-Bead extracts identified all of 26 of the direct smear-positive samples either with the same microscopy score, or in 19 of 27 samples, with an increased microscopy score, which aided microscopy detection. In addition, microscopy of the TB-Bead extracts identified 10 additional positive samples compared to direct smear which represented a statistically significant increase in case finding of 38% ($p = 0.002$) compared to direct smear.⁽¹⁷⁶⁾ Another study⁽¹⁷⁷⁾ found that the correlation between the TB-Beads protocol and indirect microscopy was 96.1% (124/129). The TB-Beads protocol was 89.4% (76/85) sensitive compared to culture and 77.8% (77/99) sensitive compared to clinical diagnosis.⁽¹⁷⁷⁾ However another study⁽¹⁷⁸⁾ found that concentration using the magnetic beads was less effective than centrifugation. These beads may aid case finding in busy laboratories particularly those that lack centrifuges, but require further evaluation.

An additional problem associated with the use of smear microscopy as a diagnostic is that serial sputa are required resulting in repeated visits to the healthcare facility for patients if they are unable to expectorate two spot

specimens on their initial visit to the clinic in countries which have adopted a frontloaded ('same day' or 'one-stop' approach) and for subsequent collection of their microscopy results.^(28, 174) Patients in rural areas may have to walk for hours to reach a clinic where laboratory services are available.⁽¹⁷⁹⁾ Patient dropout or loss to follow-up is a significant problem.⁽²⁸⁾ MacPherson *et al.* reported that pre-treatment loss to follow-up in low- and lower middle-income countries varied from 4-38%.⁽⁸⁸⁾

The International Union against Tuberculosis and Lung Disease recommendation for laboratory output of microscopy slides is 20-25 slides per technician per working day.⁽¹⁸⁰⁾ Due to overloading of the diagnostic facilities and lack of staff, most laboratory workers, especially in developing countries, process an excess of this number of slides or have to combine smear examination with other diagnostic procedures, resulting in a lower quality of the diagnostic service. In high prevalence settings, technicians spend less than the recommended time examining smears because of the high laboratory workload. A study⁽¹⁸¹⁾ conducted in Kenya demonstrated that decreasing the number of smears examined (from three to two) for the detection of new PTB cases as well as the positivity threshold to define a positive case (≥ 4 AFB/100 high-power field (HPF) instead of ≥ 10 AFB/100 HPF) resulted in increased sensitivity (96.1% versus 82%) whilst leading to a reduction of patient visits to a clinic and the laboratory workload. The reduction of the number of smears likely resulted in more time available for examination of the smears and hence impacted positively on the quality of smear microscopy as well as the number of smear-positive cases detected.⁽¹⁸¹⁾

In resource-poor countries, many smear microscopy laboratories are single rooms, understaffed, with poorly maintained microscopes and some of these laboratories lack consistent supplies of electricity and clean water.^(28, 95) Mundy *et al.*⁽¹⁸²⁾ reported in 2000 that there was approximately one

microscope per 100,000 population in Malawi, with almost one-half of these not in use or in need of repair. There are few opportunities for the training of staff and little staff capacity to handle high-volume workloads.⁽²⁸⁾ Quality assurance programs including quality control and external quality assessments are often lacking.⁽²⁸⁾

Furthermore, smear microscopy cannot identify drug-resistant strains. It cannot differentiate between *M. tuberculosis* and NTM. It may fail to detect extrapulmonary disease (for which invasive sampling to obtain lymph node aspirates, cerebrospinal fluid, or other specimens may be required).⁽⁸⁶⁾

Fluorescence microscopy was introduced in the 1930s⁽¹⁸³⁾ in an attempt to improve the sensitivity of smear microscopy. This method of microscopy increases the probability of detecting AFB, especially if the sputum contains few bacilli, and hence improves the sensitivity of microscopy in HIV-infected patients.⁽⁷²⁾ Fluorescent microscopy (auramine-based staining) is on average 10% (95% CI [5,15]) more sensitive than direct Ziehl-Neelsen (ZN) staining.⁽¹⁸⁴⁾ A systematic review⁽¹⁸⁴⁾ of 43 studies that used FM showed that on average, in comparison with ZN microscopy, FM showed a 10% increase in sensitivity and 9% incremental yield, and this improvement was not affected by HIV status. For HIV-infected patients, two studies reported between 26% and 100% increases in the detection of AFB using FM.⁽¹⁸⁴⁻¹⁸⁶⁾ Fluorescence microscopy can greatly reduce the time needed for examination of smears.⁽¹⁸⁷⁾ About fifteen times as many fields of view can be scanned by FM as by conventional microscopy in the same time period.^(72, 133)

The use of FM in resource-constrained settings is limited by high investment and maintenance costs.⁽⁷²⁾ Fluorescence microscopes are considerably more expensive than light microscopes, and the light bulbs need to be replaced

after 200 hours of use.⁽⁷²⁾ Other difficulties are the need for a reliable electricity supply and the presence of naturally fluorescent particles in sputum that can be confused with AFB.⁽⁷²⁾

Light-emitting diodes have been adapted to offer FM without the associated costs.⁽¹⁸⁸⁻¹⁹⁰⁾ The diodes are very durable, do not require warm-up time, and do not contain toxic products. Importantly, they are reported to perform equally well without a darkroom.⁽¹⁸⁹⁾ These qualities make them attractive for use in low- and middle-income countries, and they have performed well in evaluations in these settings.⁽¹⁹¹⁻¹⁹⁵⁾ The efficacy of LED microscopy was assessed by the WHO⁽¹⁹⁶⁾ and showed that “the accuracy of LED microscopy was equivalent to that of international reference standards; it was more sensitive than conventional ZN microscopy, and it had qualitative, operational, and cost advantages over both conventional fluorescence and ZN microscopy”. Based on these findings, the WHO recommends that conventional FM be replaced by LED microscopy and that LED microscopy be phased in as an alternative for conventional ZN light microscopy.⁽¹⁹⁶⁾

A recent publication has shown that SYBR®Gold (Life Technologies, Eugene, Oregon, USA) shows great promise as a stain.⁽¹⁹⁷⁾ This proprietary asymmetrical cyanine dye was selected by Ryan *et al.*⁽¹⁹⁷⁾ for acid-fast staining purposes based on both its target abundance and specificity (being DNA and RNA), and its unique fluorescence spectral characteristics. SYBR®Gold exhibits >1000-fold fluorescence enhancement on binding to double- or single-stranded DNA or to RNA, and provides low fluorescence background and strong fluorescence intensity of stained material than do other nucleic acid dyes.⁽¹⁹⁰⁾ Ryan *et al.*⁽¹⁹⁷⁾ showed that the new stain detected 99% of *M. tuberculosis* in both actively replicating aerobic and non-replicating hypoxic cultures whilst transmission light microscopy with Ziehl-Neelsen fuchsin

and FM with auramine-O or auramine-rhodamine detected only 54-86% of TB bacilli.

Recent developments of automated AFB smear microscopy systems may allow more near-patient decentralisation of microscopy as a diagnostic platform for TB with improved sensitivity and case detection.⁽¹⁹⁸⁾ Automation could enhance the consistency of results and efficiency of the process, and reduce the human resources required.⁽¹⁴⁴⁾ Automated staining systems include the RAL Stainer System (RAL Diagnostics, Martillac, France) and the Aerosprayt TB Series 2 (ELITechGroup Biomedical Systems, Puteaux, France).⁽¹³⁹⁾ TBDx (Signature Mapping Medical Sciences, Inc., Leesburg, Virginia, USA) is a system that loads stained slides onto a fluorescent microscope, focuses automatically and captures images digitally, and then classifies the smears as positive or negative, based on computerised algorithms.⁽¹⁹⁸⁾ In a proof-of-concept study⁽¹⁹⁸⁾ of 981 smears, the sensitivity (using culture as the reference standard) of the system was 75.8%, but specificity was low (43.5%) due to the fact that a large number of smears classified by TBDx as positive were from culture-negative sputum. Using the TBDx, Ismail *et al.*⁽¹⁹⁹⁾ similarly reported a sensitivity of 80% (87/109; 95% CI [71, 87]) and a specificity of 78.9% (710/900; 95% CI [76.1, 81.5]) when including one putative AFB as a positive result and using mycobacterial culture as the reference standard.

The adaptation of standard mobile phone cameras to microscopy, with or without image analysis is another possible advance. Potential advantages are portability, affordability, and availability.⁽¹⁴⁴⁾ Proof of principle has been achieved - Breslauer *et al.*⁽²⁰⁰⁾ reported the development of a high-resolution microscope attachment for mobile phone cameras that was capable of fluorescence and brightfield imaging. The authors demonstrated its ability to detect AFB in auramine O-stained sputum smears using a 3.2 megapixel

phone camera as well as the capacity of the system for automated numeration of bacilli.⁽²⁰⁰⁾ This CellScope prototype (designed at the University of California [Berkley, California, USA] and manufactured by The Pilot Group [Monrovia, California, USA]) was subsequently used by individuals with limited previous microscopy experience to perform LED FM on 525 slides.⁽²⁰¹⁾ Using a culture-based reference standard, the sensitivity (130/207, 63%) and specificity (271/318, 85%) of the CellScope were lower than for conventional LED FM performed by experienced technicians, but were within the pre-specified margins of non-inferiority.⁽²⁰¹⁾

5.1.5.2 Mycobacterial culture

Sputum culture is the reference standard for the diagnosis of PTB and is recommended for that purpose in all developed countries. In resource-poor settings, culture is recommended selectively and is mainly used for surveillance of drug susceptibility, to confirm treatment failure and relapse, and in PTB patients with repeated negative smear results.⁽⁷²⁾

TB culture is more sensitive than microscopy. Whilst the level of detection of microscopy is 5,000-10,000 bacilli/mL of sputum, culture can detect about 100 colony forming units (CFU)/mL [10-50 CFU/mL for liquid culture].^(90, 164) In a study⁽²⁰²⁾ of HIV-infected TB patients in Khayelitsha, South Africa, 49% of a consecutive sample of 109 patients had negative smears on direct microscopy but their sputum cultures were positive. Culture permits identification of the mycobacterial species responsible for disease as well as enables DST of isolates.

The disadvantages are the cost (significantly more expensive than smear microscopy), extended incubation times (due to the slow replication rate of

the organism), the need for specialised equipment, highly trained personnel, biosafety infrastructure, and a reliable supply of water and electricity. Growth of oropharyngeal flora (bacteria and yeasts) may contaminate and hamper the culture of mycobacteria. As a result, sputum samples are decontaminated with a variety of agents (acids, alkalis, detergents) in an attempt to destroy the contaminants.^(172, 203) Current bacterial decontamination methods probably reduce viable *M. tuberculosis* counts by at least 1 log, and thus may affect sensitivity of culture.⁽⁵¹⁾ In experienced laboratories, approximately 5–10% of specimens fail to yield results because of contamination.⁽¹⁸⁸⁾ Up to 15–20% of adults with PTB, whose diagnosis has been based on clinical, radiographic, histopathological findings, and response to antituberculosis treatment, have negative sputum cultures.⁽⁵³⁾

Culture is technically complex, and quality control measures are also required to prevent cross-contamination between patient specimens. The specificity of culture is affected by contamination since manipulations in the laboratory can result in transfer of bacteria from positive to negative samples.⁽⁷²⁾ Even in microbiology laboratories with the best anti-contamination procedures, one to four per cent of positive cultures might be false-positives.⁽¹³³⁾

Conventional culture that uses a solid growth medium made from egg or agar is five to 10 times more costly per sample than smear microscopy.⁽¹³³⁾ Growth of TB on traditional solid media such as Lowenstein-Jensen (LJ) or Ogawa medium is significantly longer than in liquid media [Figure 10]. Solid culture typically takes between four to eight weeks for results.⁽¹⁹⁷⁾ Whilst liquid culture is more rapid than solid culture, it is more prone to contamination.⁽²⁰⁴⁾ The long delays involved in obtaining culture results mean that they are usually not clinically useful early in the diagnostic process.⁽²⁰⁵⁾

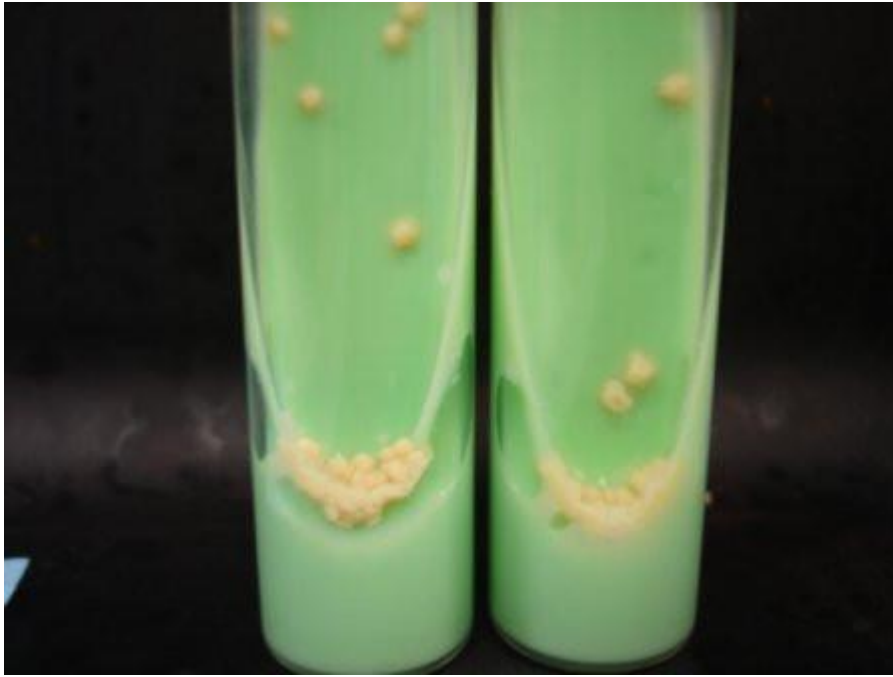


Figure 10. *M. tuberculosis* colonies on Lowenstein-Jensen medium.⁽²⁰⁶⁾

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Modern liquid media and accurate growth detection systems improve the sensitivity and greatly shorten the time needed for growth to be detected.⁽⁷²⁾ The mycobacterial growth incubator tube (MGIT) [Becton Dickinson and Company, Sparks, Maryland, USA] is one of the most studied culture methods [Figure 11].⁽⁷²⁾ The mean incubation time in MGIT is shorter and ranges from 8-16 days, including in HIV-infected TB patients, as compared with 20-26 days in conventional culture (LJ) media.^(207, 208) The same infrastructure and technical expertise are needed as for the conventional culture method, and the MGIT is costly to install which restricts its use, especially in peripheral facilities or resource-constrained settings.



Figure 11. Mycobacterial Growth Indicator Tube (MGIT) machine and technologist loading machine with MGIT tube.⁽²⁰⁹⁾

Rapid species confirmation of positive cultures is possible through the use of rapid antigen detection tests for speciation.⁽²¹⁰⁾ There are currently three manufacturers of these rapid tests, which detect the TB-specific protein MPT64 in a lateral flow format (e.g., Capilia-TB, TAUNS, Numazu, Japan).^(210, 211) In 2007, the WHO released a policy statement⁽²¹²⁾ on the use of liquid culture systems and on species confirmation through antigen detection. This statement recommended the use of TB liquid culture and DST in low-income settings.⁽²¹²⁾ The statement also indicated that it is imperative that all mycobacterial isolates be speciated at least to the level of *M. tuberculosis* complex vs. NTM.⁽²¹²⁾

Most of the resource-poor regions of the world with a high burden of TB have very few reference laboratories capable of reliably performing TB culture and DST.⁽²⁸⁾ Currently, TB culture laboratories in resource-poor countries often lack adequate infrastructure and have inadequate or outdated equipment and poor biosafety measures, with a scarcity of human and financial resources.^(28, 93) The WHO issued a policy⁽²¹²⁾ which recommends phased implementation of the liquid culture systems as part of a country-specific comprehensive plan for laboratory capacity strengthening, and

addresses key issues, including biosafety, staff training, maintenance of infrastructure and equipment, specimen transport, and reporting of results.

5.1.5.3 Microscopic observation drug susceptibility assay

The microscopic observation drug susceptibility (MODS) assay has been recommended by the WHO as an affordable and effective alternative to the existing reference standard (liquid mycobacterial culture).⁽²¹²⁾ This test is rapid, economical and a highly sensitive/specific method for the detection of *M. tuberculosis* and DST directly from sputum.⁽²¹³⁾

The test uses 24-well plates with four wells for a single patient specimen: two wells are drug-free, while the other two wells contain RIF and INH (Figure 12). After inoculation, the plates are sealed in ziplock plastic bags and then incubated.⁽²¹³⁾ *M. tuberculosis* grows rapidly in a liquid medium, and a diagnosis is made using morphological characterisation patterns specific to *M. tuberculosis* (cording) when viewed under an inverted light microscope. Despite this, MODS is not widely used in resource-limited settings due to concerns regarding biosafety and efficiency for handling large numbers of samples.⁽²¹⁴⁾ MODS also requires manual reading of each individual well by trained laboratory professionals, which requires both time and additional human resources.^(214, 215) Moreover, there is also concern about the assay's ability to differentiate *M. tuberculosis* and NTM.⁽²¹⁵⁾



Figure 12. Microscopic observation drug susceptibility (MODS) test kit.⁽²¹⁶⁾

A multicentre study performed in Honduras and Brazil⁽²¹⁷⁾, which included 1,639 respiratory specimens obtained from 854 study subjects, found that MODS sensitivity was 97.5% (95% CI [95.7, 98.6]), and specificity was 94.4% (95% CI [93.1, 95.2]). Median times to detection were 21 days (interquartile range [IQR], 17–25 days) and 7 days (IQR, 5–10) for culture on LJ medium and for the MODS assay, respectively ($p < 0.01$).⁽²¹⁷⁾ For 64 specimens cultured using the MGIT 960 automated system [Becton Dickinson and Company, Sparks, Maryland, USA], median time to growth was similar for the MODS assay (7 days; IQR, 7–10 days) and the MGIT 960 automated system (8 days; IQR, 6–11.5 days; $p = 0.16$).⁽²¹⁷⁾

A few studies have evaluated the accuracy of MODS in HIV-infected patients. One such study performed by Ha *et al.*⁽²¹⁸⁾, who evaluated the MODS assay for the early diagnosis of TB in 307 HIV-infected patients presenting to Pham Ngoc Thach Hospital for Tuberculosis and Lung Diseases in southern Vietnam, found that the sensitivity of MODS using a microbiological gold standard (either smear- or culture-positive) was 87% and specificity was 93%. The authors reported that the median times to detection using MODS and MGIT for smear-positive samples were eight and 11 days, respectively, and they were 11 and 17 days, respectively, for smear-negative samples.⁽²¹⁸⁾

Wang *et al.*⁽²¹⁹⁾ evaluated an automated MODS (Auto-MODS) assay which utilises 1.5-mL screw-cap tubes instead of the well plates; a computer-assisted digital camera to automatically and consecutively take images of each tube used in Auto-MODS, and a low-speed centrifuge which removed large particles in the liquid before inoculation of each sample into the Auto-MODS tube. The images taken by the digital camera were automatically transferred to a computer and were visually read by the laboratory technician directly on the computer screen.⁽²¹⁹⁾ This evaluation of the automated MODS assay in Thailand reported that of the 221 true-positive samples, Auto-MODS identified 212 as positive and 9 as negative (sensitivity, 95.9%; 95% CI [92.4, 98.1]).⁽²¹⁹⁾ Of the 139 true-negative samples, Auto-MODS identified 135 as negative and 4 as positive (specificity, 97.1%; 95% CI [92.8, 99.2]).⁽²¹⁹⁾ The median time to culture positivity was 10 days (IQR, 8-13 days).⁽²¹⁹⁾

5.1.5.4 Molecular-based assays

Nucleic acid amplification tests (NAATs) are assays that amplify nucleic acid sequences of a bacterium to a concentration that they can be detected by gel-based, biochemical or fluorescent methods. Whilst TB diagnostic tests in the past have relied on culture to increase the number of bacilli, tests such as NAATs, which detect specific nucleic acid sequences or molecules present in diagnostic specimens, can reduce the overall turnaround time (TAT) for the laboratory diagnosis of TB by at least two to four weeks compared to conventional growth detection.^(28, 164) The test formats can be separate and manual, or fully integrated and automated, or somewhere in between.⁽¹⁴⁴⁾ NAATs have revolutionised the diagnosis of infectious diseases and represent a powerful strategy for early and accurate detection of pathogens. Manual NAATs include in-house and commercial tests, and usually employ polymerase chain reaction (PCR) or ligase chain reaction methods to amplify

target TB genes such as Insertion Sequence (IS) 6110, *mpb64*, *mtp40*, IS986, and heat shock protein 65(*hsp65*).⁽²²⁰⁻²²⁴⁾ These assays are performed directly on patient samples or on culture colonies and results can be available on the same day.

Molecular diagnostics in TB have enabled direct detection of *M. tuberculosis* complex in clinical specimens, identification of other mycobacteria, detection of drug resistance of *M. tuberculosis*, DNA typing to differentiate reactivation of disease from exogenous reinfection and to track transmission, and internal laboratory contamination.⁽²²⁵⁾ In general, NAATs have better sensitivity than smear microscopy for the diagnosis of TB. Compared with culture, NAATs can detect the presence of *M. tuberculosis* in a specimen weeks before culture for 80–90% of patients suspected to have PTB whose TB is ultimately confirmed by culture.⁽²²⁶⁻²²⁸⁾

Commercial and in-house manual NAATs have many limitations, including low sensitivity in sputum smear-negative TB, and the need for PCR laboratory capacity, skilled personnel, specific infrastructure and biosafety requirements. Additional disadvantages of this testing format are false-negative results due to the presence of PCR inhibitors (enzymes in sputum or other specimens that inhibit the amplification reaction) as well as paucibacillary disease (low bacterial load) below the level of detection of the NAAT assay.⁽²²⁶⁾ False-positives tend to arise from contamination when amplicons may be inadvertently transferred from one specimen to another.⁽²²⁶⁾ Sample contamination with genomic DNA may occur from the environment, during sample preparation, and there may be contamination of a sample with amplified DNA from a previous PCR reaction. NAATs are also not useful for monitoring treatment progress since they can detect non-viable bacteria and give false-positive results.⁽²²⁸⁾

Many commercial NAATs are available, and hundreds of studies on their efficacy have been published in recent years. Most tests perform well in sputum smear-positive specimens, but have suboptimal sensitivity in sputum smear-negative samples. The cost of commercial tests ranges between US \$10 (special pricing of GeneXpert®MTB/RIF assay for public sector laboratories in high burden countries) and \$50, whereas in-house tests are approximately US \$15.⁽²²⁷⁾ Studies of commercial NAATs (AMTD, E-MTD, Roche Amplicor, Cobas Amplicor, BDProbe-Tec, BDProbeTec-ET, Lightcycler test) on sputum samples generally have reported sensitivities of 90% to 100% and specificities of 71% to 96% in sputum smear-positive samples, but significantly lower sensitivities of 22% to 89% and specificities of 97% to 99% in sputum smear-negative samples.^(67, 226, 229-237)

Earlier molecular technologies were restricted to highly sophisticated reference laboratories partly due to their costs and complexity, and because their sensitivity did not allow them to screen for resistance associated mutations directly in clinical specimens. Therefore, many TB and MDR-TB patients could not benefit from the advantages of these techniques since most patients did not have access to these reference laboratory services. A breakthrough came when molecular line probe assays (LPAs) began to be used directly on clinical specimens of TB and MDR-TB patients, resulting in a paradigm shift of setting up cultures only when resistance-associated mutations are detected.⁽²³⁸⁾

5.1.5.4.1 Line probe assays

Novel technologies for rapid detection of TB drug resistance have become a priority in TB research and development.⁽²³⁹⁾ Line probe assays are DNA strip-based tests that can detect specific gene markers associated with RIF resistance alone or in combination with INH resistance.⁽¹⁸⁸⁾ Briefly, LPA technology involves the extraction of DNA from cultured mycobacterial isolates or directly from clinical specimens followed by PCR amplification of the resistance-determining region of the gene.⁽²³⁹⁾ Following amplification, the labelled PCR products are hybridised with oligonucleotide probes (short sequences of nucleotides that match a specific region of DNA allowing detection of that specific DNA sequence) which are immobilised on a strip.⁽²³⁹⁾ Captured labelled hybrids are detected by colourimetric development that allows for lines to be visualised at the site of probe binding [Figure 13].^(188, 239)

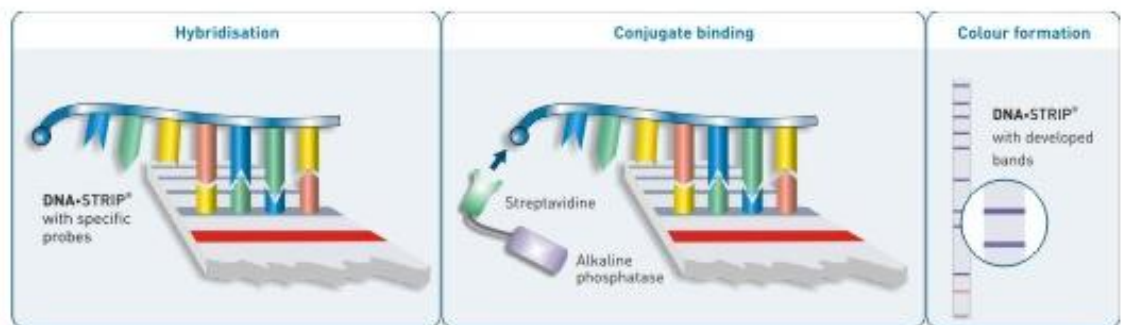


Figure 13. GenoType MTBDRplus assay, showing the hybridisation and colourimetric development of lines.⁽²⁴⁰⁾

Line probe assays enable detection of the presence of *M. tuberculosis* complex, as well as the presence of wild-type and mutation probes for resistance. If a mutation is present in one of the target regions, the amplicons will not

hybridise with the relevant probe. Mutations are therefore detected by lack of binding to wild-type probes, as well as by binding to specific probes for the most commonly occurring mutations (Figure 14).

The *rpoB* gene, which encodes the β -subunit of RNA polymerase, consists of an 81 base pair hotspot region from codons 507 to 533. This is also known as the rifampicin resistance determining region (RRDR).⁽²³⁸⁾ Point mutations at codons 526 or 531 are associated with high level RIF resistance, whereas mutations in codons 511, 516, 518, 522 and 533 cause low level resistance.⁽²³⁸⁾ More than 96% of RIF-resistant strains contain a mutation in the RRDR.^(238, 241, 242)

Mutations in the mycobacterial catalase-peroxidase gene (*katG*) account for 42% to 58% of INH-resistant clinical isolates.^(238, 241, 242) Altered catalase-peroxidase (particularly as a result of the Ser315Thr mutation) provides high-level resistance to INH, whilst isolates that carry mutations in other genes such as *inhA* and *kasA* exhibit low-level resistance to INH.^(238, 241) *InhA*, the fatty-acid enoyl-acyl carrier protein reductase, is an enzyme involved in synthesis of mycolic acids.^(243, 244)

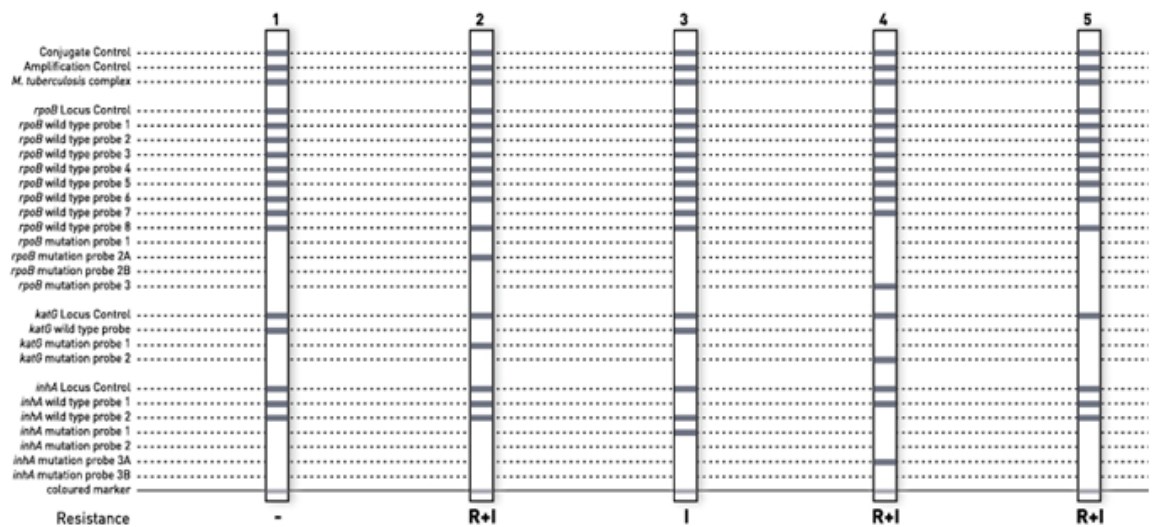


Figure 14. Examples of possible line probe assay results (GenoType MTBDR_{plus} line probe assay version 2.0).⁽²⁴⁵⁾

Key: *rpoB* (codes β -subunit of the bacterial RNA polymerase), mutations of this gene confer rifampicin (RIF) resistance; *katG* (catalase-peroxidase gene), mutations confer resistance to isoniazid (INH); *inhA*, gene which confers resistance to INH and ethionamide.

LPA 1- Wild-type (WT) strain.

LPA 2- RIF and INH resistant strain: *rpoB* WT probe 7 missing and *rpoB* mutation (MUT) probe 2A present; *katG* WT probe missing and *katG* MUT 1 probe present.

LPA 3- INH resistant: *inhA* WT probe 1 missing; *inhA* MUT probe 1 present.

LPA 4- RIF and INH resistant: *rpoB* WT 8 missing and *rpoB* MUT probe 3 present; both *katG* WT probe 1 and *inhA* WT probe 2 missing with corresponding mutation probes detected in *katG* MUT 2 and *inhA* MUT 3A.

LPA 5- RIF and INH resistant: *rpoB* WT 7 missing with unknown MUT present; no *katG* WT present with unknown *katG* MUT present.

There are currently two LPAs available commercially: the INNO-LiPA®Rif TB kit (Innogenetics NV, Gent, Belgium) and the GenoType® MTBDR_{plus} assay (Hain Lifescience, GmbH, Nehren, Germany). A major advantage of the LPAs is that they can be performed directly on clinical specimens, such as sputum.^(246, 247) The standard TAT for reporting LPA results is two to three

days.⁽²³⁹⁾ Detailed costing data from South Africa showed that by using the LPAs in routine diagnostic algorithms, the reduction in cost amounted to between 30% and 50% compared to conventional DST methods.⁽²⁴⁸⁾

Systematic reviews and meta-analyses of the performance of molecular LPAs compared to conventional DST methods showed that LPAs are highly sensitive ($\geq 95\%$) and specific ($\geq 98.7\%$) for the detection of RIF resistance in culture isolates of *M. tuberculosis*.^(239, 249, 250) However, a systematic review and meta-analysis by Morgan *et al.*⁽²⁴⁹⁾ showed that when performed directly on sputum specimens, the INNO-LiPA® sensitivity for detection of RIF resistance ranged from 80% to 100% with a specificity of 100%. This suggests that the INNO-LiPA® is a highly sensitive and specific test for the detection of RIF resistance in culture isolates. Ling *et al.*⁽²⁵⁰⁾, who performed a systematic review and meta-analysis on the GenoType®MTBDR and MTBDR*plus* assays, showed a pooled sensitivity of 98.1% (95% CI [95.9, 99.1]) and specificity of 98.7% (95% CI [97.3, 99.4]) which was consistent across all groups, assay versions and specimen types (i.e. clinical specimen vs. culture isolate). These authors however showed that the accuracy for INH resistance was variable and inconsistent, with a lower sensitivity of 84.3% (95% CI [76.6, 89.8]) and specificity of 99.5% (95% CI [97.5, 99.9]).⁽²⁵⁰⁾ The evidence suggests that the GenoType® assays have excellent accuracy for RIF resistance, whilst the sensitivity for testing of INH resistance is modest and variable.⁽²⁵⁰⁾ Based on the available evidence and expert opinion, the WHO endorsed the use of LPAs for rapid screening of patients at risk of MDR-TB.⁽²³⁹⁾

In March 2016, the WHO issued updated policy recommendations on the use of the second-line LPA, the GenoType®MTBDRsl (Hain Lifescience, GmbH, Nehren, Germany).⁽²⁵¹⁾ This LPA is used for the rapid determination of genetic mutations associated with resistance to fluoroquinolones and the second-line injectable drugs, including kanamycin, amikacin, and

capreomycin.⁽²⁵¹⁾ The assay format is similar to the GenoType®MTBDRplus assay. The assay can be performed directly using a processed sputum specimen or indirectly using DNA isolated and amplified from mycobacterial culture of the patient's specimen.⁽²⁵¹⁾ There are two versions of the assay. Version 1.0 detects *M. tuberculosis* complex resistance to fluoroquinolones (*gyrA* gene), second-line injectables (*rrs* gene), and ethambutol (*embB* gene). Version 2.0 differs from Version 1.0 by detecting additional resistance genes for fluoroquinolones (*gyrA* and *gyrB*) and second-line injectables (*rrs* and *eis* promoter region), whilst ethambutol resistance testing has been removed. In addition, Version 2.0 may be performed on smear-positive and smear-negative specimens and culture isolates. Based on evidence from 29 studies on the assay (which included 26 studies evaluating the MTBDRsl version 1.0 assay and three (one published, two unpublished) evaluating the MTBDRsl version 2.0 assay [Table 3], the WHO⁽²⁵¹⁾ recommended that the assay may be used as the initial test, instead of phenotypic culture-based DST, to detect resistance to fluoroquinolones and second-line injectables in patients with confirmed rifampicin-resistant TB or MDR-TB (conditional recommendation; moderate certainty for direct testing of sputum specimens; low certainty for indirect testing of *M. tuberculosis* cultures).

Table 3. Summary of results of the WHO Policy Guidance document evidence base showing the accuracy of the MTBDRs/ assay version 1.0* for fluoroquinolone and second-line injectable drug resistance and XDR-TB, indirect and direct testing (smear-positive specimens), phenotypic culture-based DST standard reference.⁽²⁴⁶⁾

	Number of studies	Number of participants	Pooled sensitivity [95% CI]	Pooled specificity [95% CI]
Fluoroquinolone resistance				
Indirect testing	19	2,223	85.6% [79.2, 90.4]	98.5% [95.7, 99.5]
Direct testing	9	1,771	86.2% [74.6, 93.0]	98.6% [96.9, 99.4]
Second-line injectables				
Indirect testing	16	1,921	76.5% [63.3, 86.0]	99.1% [97.3, 99.7]
Direct testing	8	1,639	87.0% [38.1, 98.6]	99.5% [93.6, 100.0]
Diagnostic accuracy to detect XDR-TB				
Indirect testing	8	880	70.9% [42.9, 88.8]	98.8% [96.1, 99.6]
Direct testing	6	1,420	69.4% [38.8, 89.0]	99.4% [95.0, 99.3]

*For MTBDRs/ version 2.0, the data were either too sparse or too heterogeneous to combine in a meta-analysis or to compare indirect and direct testing.

Direct testing implies testing directly on clinical specimens.

Indirect testing implies testing on cultured isolates of *M. tuberculosis*.

5.1.5.4.2 Xpert®MTB/RIF assay

The Xpert®MTB/RIF assay is based on an automated, hemi-nested, real-time PCR and molecular beacon technology that can simultaneously detect *M. tuberculosis* as well as RIF resistance within two hours of commencing the test directly on sputum samples [Figure 15].⁽²⁵²⁾ The assay employs five molecular beacons (unique nucleic acid hybridisation probes), each labelled with a coloured fluorophore responding to a specific target sequence within the *rpoB* gene.⁽²⁵³⁾ The generation of all five fluorescent colours following PCR amplification indicates the presence of RIF-susceptible *M. tuberculosis*, whilst any mutation that prevents the binding of the respective molecular beacon results in the absence of colour and indicates RIF resistance.⁽²⁵³⁻²⁵⁶⁾ Although both standard and real-time PCR follow a similar procedure, there are many advantages to real-time PCR. Whilst standard PCR measures product during the plateau phase, real-time PCR measures the product during the exponential phase which is a more effective measure.⁽²⁵⁷⁾ Standard PCR requires post-PCR analysis to identify the product either by size or sequence, whereas real-time PCR eliminates this.⁽²⁵⁷⁾ A further advantage of real-time PCR is that the entire process from amplification to analysis is performed in the same tube, thus decreasing the possibility of contaminating the end-product.⁽²⁵⁷⁾

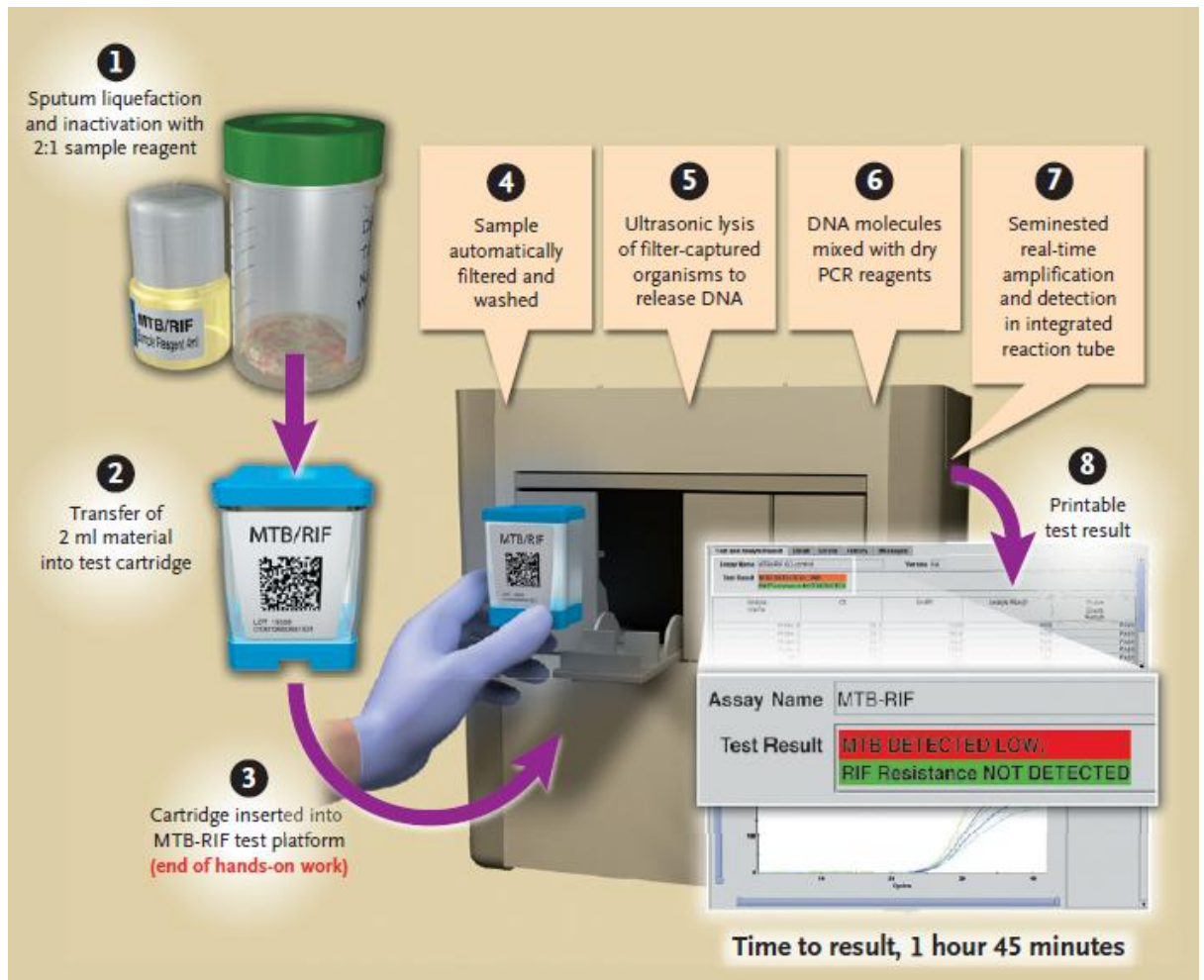


Figure 15. Assay procedure for the Xpert®MTB/RIF test.⁽²⁵⁸⁾

Reproduced with permission from The New England Journal of Medicine; Vol. 363, No. 11; Boehme CC, Nabeta P, Hilleman D, Nicol MP, Shenai S, Krapp F, et al; Rapid molecular diagnosis of tuberculosis and rifampicin resistance; Pages 1005-1015; doi: 10.1056/NEJMoa0907847; Copyright 2010 Massachusetts Medical Society.

The limit of detection (95% reliability for detection) of the Xpert®MTB/RIF assay is 131 CFU/mL (95% CI [106, 176]) of sputum.⁽²⁵⁴⁾ This result contrasts with the limit of detection of automated mycobacterial liquid culture, which is about 10–50 CFU/mL, and with that of smear microscopy, which is about 10,000 CFU/mL.⁽¹⁶⁴⁾

Advantages of the assay include the integration of sample processing and PCR amplification into a self-contained cartridge making the test easy to use, the rapidity of obtaining a result (two hours), minimal biosafety risks to staff working with the specimen due to the tuberculocidal action of the sample reagent preparation, lack of need for formal laboratory training of the operator, and the ability to run the test at the point-of-care (POC).^(90, 252, 259) In addition, the Xpert® detects both *M. tuberculosis* as well as RIF resistance; has a low risk of cross-contamination; is multifunctional and can be used for other diagnostic test cartridges such as HIV viral load; and potentially could be used to test a broad range of specimens, such as cerebrospinal fluid and fine needle aspirates.^(90, 260) Xpert® guided management can hypothetically reduce over-treatment even in a low-burden setting as shown by Davis *et al.*⁽²⁶¹⁾

This technology represents a major landmark in TB diagnostics, but due to the high cost of testing; need for electrical supply, temperature and humidity controls, sophisticated equipment; yearly servicing and calibration of the instrument; this test is likely to remain confined to the laboratory.^(259, 262, 263) Denkinger *et al.*⁽⁹⁵⁾, who surveyed at least three TB experts from 22 high burden countries regarding the landscape of peripheral microscopy centres, showed that many of the countries lacked the infrastructure, equipment and skills to implement molecular tests as replacement of smear microscopy (Figure 16). These authors acknowledge that their data does not accurately reflect every microscopy centre in each country; rather it reflects the “typical” microscopy centre. In addition, they acknowledge that the results may also be overoptimistic as the presence of equipment does not guarantee its use or functionality. Other disadvantages include the limited duration of storage of specimens at room temperature (three days), the short half-life of reagent cartridges, and that the operator must be computer literate. This assay is also not able to assess resistance to drugs other than RIF. Although relatively rapid, the TAT remains a challenge for same-day diagnosis and treatment in

overcrowded health facilities.⁽⁹⁰⁾ Results of operational research into POC implementation showed that the TAT for sample processing was often more than two hours and that failure to link results to patients on the same day was an unforeseen difficulty.⁽²⁶⁴⁾

	Country	Environment		Infrastructure			Available equipment							
		Temp.	Humidity	Power	Water	Security	Gloves	N95	Pipettes	Refrigerator	Incubator	Centrifuge	Water bath	Hood
Non-BRICS	Congo													
	Zimbabwe													
	Mozambique													
	Ethiopia													
	Afghanistan													
	Myanmar													
	Uganda													
	Tanzania													
	Kenya													
	Bangladesh													
	Cambodia													
	Nigeria													
	Pakistan													
	Vietnam													
	Philippines													
Indonesia														
Thailand														
BRICS	India													
	China													
	South Africa													
	Brazil													
	Russia													

	Country	Skills			Communication			QA	Stockouts	Waste	Current testing	
		Pipetting	PCR tests	Computer	Landline	Mobile	Internet	established		established	ZN	FM
Non-BRICS	Congo											
	Zimbabwe											
	Mozambique											
	Ethiopia											
	Afghanistan											
	Myanmar											
	Uganda											
	Tanzania											
	Kenya											
	Bangladesh											
	Cambodia											
	Nigeria											
	Pakistan											
	Vietnam											
	Philippines											
Indonesia												
Thailand												
BRICS	India											
	China											
	South Africa											
	Brazil											
	Russia											

Yes/present

Maybe

No/not present

Unsure/question not answered

Figure 16. Characteristics of peripheral microscopy centres in 22 high

burden countries.⁽⁹⁵⁾ Reproduced with permission of the European Respiratory Society

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Although the Xpert®MTB/RIF assay represents a significant step forward toward developing a rapid accurate test that can be used at the peripheral

health centre level, it still misses one-fourth to one-third of patients in the critical smear-negative group (when one sample is tested).⁽⁶⁷⁾ The Xpert®MTB/RIF assay detects both live and dead bacteria and so might give a positive result despite the patient being culture-negative making it unsuitable currently for monitoring response to treatment and in patients recently treated for TB.^(265, 266)

The multi-country assessment done by FIND⁽²⁵⁸⁾, which was published in 2010, enrolled 1,730 patients suspected of having drug-sensitive or drug-resistant TB at five study sites in South Africa, Peru, Azerbaijan, and India. One direct test on sputum detected 551 (98.2%) of 561 patients with smear-positive TB and 124 (72.5%) of 171 patients with smear-negative TB.⁽²⁵⁸⁾ The test was specific in 604 (99.2%) of 609 patients without TB.⁽²⁵⁸⁾ In patients with smear-negative TB, processing one, two, or three samples was associated with sensitivities of 72.5%, 85.1%, and 90.2%, respectively.⁽²⁵⁸⁾ In all centres, the GeneXpert® machines were located within laboratories at health facilities where smear microscopy was being done.⁽²⁶⁷⁾ The assay greatly accelerated the time to diagnosis, with a median time of 0 days compared with one day for smear microscopy, 16 days with liquid culture, and 20 days with solid culture.⁽²⁶⁷⁾ For patients with smear-negative TB, the Xpert®MTB/RIF assay reduced the median time to start of treatment from 56 days (IQR, 39–81) to 5 days (IQR, 2–8).⁽²⁶⁷⁾ A recent Cochrane review⁽²⁵⁹⁾ [22 studies; 8,998 participants: 2,953 confirmed TB; 6,045 non-TB] reported a pooled sensitivity of 89% (95% Credible Interval (CrI) [85, 92]) and pooled specificity of 99% (95% CrI [98, 99]) when sputum Xpert®MTB/RIF was used as an initial test replacing smear microscopy, based on mycobacterial culture as the reference standard. As an add-on test following a negative smear microscopy result, Xpert®MTB/RIF pooled sensitivity was 67% (95% CrI [60, 74]) and pooled specificity 99% (95% CrI [98, 99]; 21 studies; 6,950 participants).⁽²⁵⁹⁾ For people with HIV infection, Xpert®MTB/RIF pooled sensitivity was 79% (95% CrI [70, 86]; seven studies; 1,789 participants), and

for people without HIV infection, it was 86% (95% CrI [76, 92]; seven studies; 1,470 participants).⁽²⁵⁹⁾

The Cochrane review⁽²⁵⁹⁾ also found that for RIF resistance detection, Xpert®MTB/RIF pooled sensitivity was 95% (95% CrI [90, 97]; 17 studies; 555 RIF resistant-positives) and pooled specificity was 98% (95% CrI [97, 99]; 24 studies; 2,411 RIF resistant-negatives). Increasing evidence has shown that the occurrence of false-positive results may be linked to the detection by the Xpert® of strains that are truly resistant to RIF, but are not detected by phenotypic culture-based DST. Van Deun *et al.*⁽²⁶⁸⁾ showed that 10-13% of RIF-resistant strains in patients who experienced first treatment failure and in relapsed patients may be missed by rapid phenotypic DST. The positive predictive value (PPV) [the proportion of cases diagnosed as RIF-resistant that are truly resistant] using the Xpert®MTB/RIF assay exceeds 90% in settings where the underlying prevalence of RIF resistance is >15%.^(253, 269) Using the Xpert®MTB/RIF assay version G4 cartridge, Osman *et al.*⁽²⁷⁰⁾ reported a PPV of 99.5% (95% CI [98.5, 100]) for RIF resistance in their study conducted in Cape Town, South Africa. In settings where RIF resistance is rare, the PPV is likely to be adversely affected, diminishing to 71.4% and 32.4% when the RIF resistance prevalence is 5% and 1%, respectively.^(253, 269) Interestingly, a recent study of the G4 cartridge in Brazil⁽²⁷¹⁾, a country with a low prevalence of RIF resistance, found a PPV of 94-95% (assuming sensitivity of 95%, specificity of 99.8% and a prevalence of RIF resistance of 3-4%). An issue of relying solely on the Xpert®MTB/RIF assay to detect RIF resistance is the assay's inability to identify mutations outside the RRDR. A recent publication by Sanchez-Padilla *et al.*⁽²⁷²⁾ highlighted this problem in Swaziland, where approximately 38 of 125 (30%) MDR strains in the 2009 national survey⁽²⁷³⁾ carried the *rpoB* I491F mutation, which confers resistance to RIF but is not detected by the Xpert®MTB/RIF assay.⁽²⁷⁴⁾ The authors concluded that Xpert®MTB/RIF testing may be unreliable in Swaziland since it may miss a substantial percentage of strains that may be resistant to

RIF and advocated for further studies in neighbouring countries to assess the prevalence of similar mutations.⁽²⁷²⁾

The Xpert®MTB/RIF assay was endorsed by the WHO in December 2010 for use as the initial TB diagnostic in HIV-associated PTB as well as in suspected drug-resistant TB.⁽²⁶⁹⁾ By June 2012, two-thirds of countries with a high TB burden and half of countries with a high MDR-TB burden had incorporated the assay into their national TB programme guidelines.⁽⁹⁰⁾ According to the WHO, as of 31 December 2014, 3,763 GeneXpert® instruments (comprising 17,883 modules) and 10,013,600 Xpert®MTB/RIF cartridges had been procured by the public sector in 116 of 145 countries eligible for concessional pricing.⁽²⁷⁵⁾ More than one million cartridges are being procured each quarter.⁽²⁷⁵⁾ Although this assay is a major step forward in TB diagnostics, issues regarding implementation in lower level of care facilities, single-source manufacture and cost remain problematic.

The high cost of this technology is the key hurdle to implementation of the test in low- and middle-income countries.^(263, 276) Initial analyses^(277, 278) of the use of the Xpert®MTB/RIF assay in countries with a high burden of TB suggest that this technology is likely to be a highly cost-effective method of TB diagnosis, although this will be setting-specific.⁽⁹⁰⁾ Cost-effectiveness does not denote affordability, however, and in the poorest countries of the world with a high TB burden, the total yearly expenditure per head on health might be little more than US \$10 to \$20.⁽⁹⁰⁾ In South Africa, for example, the national scale-up of the Xpert®MTB/RIF assay was estimated to be associated with a 53% to 57% increase in the yearly cost of the TB diagnostic programme.⁽²⁷⁹⁾ These costs would also vary depending on whether GeneXpert® machines were placed only in existing microscopy laboratories or were extended to all facilities providing TB treatment, which could increase the budget by more than 50%.⁽²⁸⁰⁾ In South Africa, for example, laboratory placement for routine

Xpert®MTB/RIF testing would require 274 instruments, whereas location at points of treatment would require 4,020 instruments with a 51% increase in cost (US \$107 million per year).⁽²⁸⁰⁾

Xpert® technology was not designed to reach lower tiers of the healthcare system or to meet all needs (e.g., it cannot detect latent *M. tuberculosis* infection or resistance against multiple drugs).⁽⁸¹⁾ A recent survey⁽²⁸¹⁾ of 22 countries with a high TB burden showed that, while a majority (86%) of these countries have a policy or algorithm for use of Xpert® technology, current implementation is mostly donor-funded, largely dependent on testing in centralised laboratories, and primarily involves patients with presumed drug resistance or HIV infection.

Most high-burden countries have an insufficient network of laboratories to effectively service the affected population.⁽²⁸²⁾ Due to feasibility, cost and logistical issues, the Xpert®MTB/RIF is likely to be implemented in centralised laboratories, leading to a critical disconnect between the patient and their test result. In a study⁽²⁸³⁾ in Cape Town, South Africa, where Xpert®MTB/RIF was initially rolled out in centralised laboratories, only 76.6% of patients diagnosed as having Xpert®-positive TB during preART screening started TB treatment. Moreover, these patients only started TB treatment after a median delay of 9 days (IQR, 6–18 days).⁽²⁸³⁾ This delay was due to an overall TAT of four days for the sample to reach the laboratory, be processed, and for the results to be issued and reach the clinic.⁽²⁸³⁾ Additional delays accrued waiting for patients to return following recall; others were found to have already died or were lost to follow-up.⁽²⁸³⁾ Another study⁽²⁸⁴⁾ of 637 patients who received Xpert® testing in Kwa-Zulu Natal, South Africa, found that amongst participants who were initiated on treatment, 28%, 40%, 21% and 8% commenced treatment within 2 weeks, 1 month, 2 months and 3

months of Xpert® testing respectively, whilst the remaining three per cent did not receive treatment.

The rollout of Xpert® has also demonstrated that new diagnostic tools do not necessarily reach additional people eligible for testing or increase the overall number of TB cases diagnosed if they are implemented within established care settings (although the GeneXpert® testing does increase the number of bacteriologically confirmed cases).^(285, 286) Using a transmission model of diagnostic testing among adults with active TB in South-East Asia, Sun *et al.*⁽²⁸⁷⁾ estimated that without Xpert®, TB incidence was projected to fall at two per cent annually, from 176 to 144 per 100,000/year over ten years. In the baseline scenario, Xpert® reduced TB incidence to 69.5 per 100,000/year (51% reduction relative to diagnosis with smear) and mortality to five per 100,000/year (82% reduction).⁽²⁸⁷⁾ However, these authors also found that empiric diagnosis dramatically affected projected impact of Xpert®-assuming 40% empiric treatment in the idealised baseline scenario blunted projected Xpert®-associated reductions in incidence and mortality from 51% to 36% and 82% to 58%, respectively.⁽²⁸⁷⁾ Importantly, Theron *et al.*⁽²⁸⁵⁾ showed in their multi-country Xpert®MTB/RIF study in sub-Saharan Africa that despite a longer delay to treatment in the microscopy group, there was no effect on the primary outcome, which was difference in morbidity according to the TB score and Karnofsky performance score in culture-positive patients who had begun treatment. The scores remained the same in the two groups at two and six months after randomisation.⁽²⁸⁵⁾ These authors also found that although the Xpert® test facilitated access to same-day initiation of treatment, the benefits did not translate into lower TB-related morbidity.⁽²⁸⁵⁾ Similarly, a randomised controlled trial⁽²⁸⁸⁾ in Zimbabwe found screening with Xpert®MTB/RIF did not reduce the rate of ART-associated TB and mortality, as compared with FM. This is in part due to the practice of prescribing antituberculosis therapy on clinical presentation and

history, despite samples testing negative for *M. tuberculosis*.⁽⁸⁹⁾ Empiric treatment for TB in non-TB cases is not however without risks-it can lead to excess mortality due to missed alternate diagnoses and medication toxicity.^(289, 290)

The literature is currently limited in relation to the use of a triage test prior to testing with Xpert®MTB/RIF.⁽²⁹¹⁾ This strategy has been proposed to reduce total diagnostic costs when compared with using Xpert® in all people presumed to have TB.⁽²⁹²⁾ At a cassette cost of US \$10 (reduced price for low-resource settings), testing large numbers of people with suspected TB will put substantial pressure on already resource-limited TB programmes in which the drugs for treatment might not always be available.⁽²⁹³⁾ It will also take a fair amount of time before the Xpert®MTB/RIF assay can be decentralised sufficiently to replace smear microscopy as a diagnostic test, particularly in geographical areas with high prevalence of MDR-TB or HIV/TB co-infections, because most of these areas are in poorly developed zones of low-income countries, with irregular availability of electricity and water, as well as a poorly developed infrastructure for uninterrupted supply of consumables and their storage.^(293, 294) The controversial question is whether TB elimination is most likely to be advanced by “distributing GeneXpert® machines to all peripheral health facilities in the world, or by investing the same amount in ensuring that health facilities have adequate laboratory set-up, i.e. well trained and paid staff, electricity, and reagents.”⁽²⁹³⁾ An important use of Xpert®MTB/RIF might be mainly in regional facilities in areas where MDR-TB is highly prevalent, rather than for large-scale diagnosis of drug-susceptible TB.⁽²⁹³⁾

5.1.5.4.3 Molecular technologies under evaluation

The 2014 UNITAID TB Diagnostics Technology and Market Landscape report summarised the technologies that have been endorsed by the WHO and described the pipeline of novel tools that are on or likely to enter the market.⁽²⁹⁵⁾ In the short term, the most impressive trend is the expansion of the range of molecular technologies that could potentially replace smear microscopy.⁽²⁹⁵⁾ Newer molecular platforms such as the GeneDrive® system [Epistem (Manchester, UK), and Becton Dickinson and Company]; the TrueLab® (Mobio, India); and the Loopamp® (Eiken, Japan) can further simplify molecular testing for TB and drug-resistant TB and may be suitable for further decentralisation of testing to healthcare centre level or to mobile diagnostic units.⁽²⁹⁶⁾

Loop-mediated isothermal amplification (LAMP) is a rapid molecular diagnostic tool that also can be implemented without the need for skilled personnel. The assay applies autocycling strand displacement DNA synthesis targeting six regions of the *gyrB* and 16S ribosomal RNA genes. The advantages of this system are its simplicity, its rapidity (results can be available within 35 to 65 minutes), its high specificity, its lack of need for costly instruments, and that the platform can be used for organisms other than *M. tuberculosis*.⁽²⁹⁷⁾ LAMP allows for the rapid amplification of genetic material (<1 hour) in a closed-tube system by heating the sample in an isothermal bath at a single reaction temperature (62°C), eliminating the need for a thermocycler.⁽⁶⁷⁾ The amplified product can be visualised by eye with good accuracy and reproducibility. LAMP is also less sensitive to inhibitors compared with standard PCR.⁽²⁹⁸⁾ Boehme *et al.*⁽²⁹⁹⁾ evaluated the diagnostic accuracy of LAMP for TB in Peru, Bangladesh, and Tanzania. Sensitivity was 98% for smear-positive sputum samples, but only 49% for smear-negative samples.⁽²⁹⁹⁾ Specificity was 99%.⁽²⁹⁹⁾ Due to the short TAT (<1 hour),

simplicity, and low cost, TB LAMP may have a place in peripheral healthcare settings despite its low sensitivity in smear-negative samples and the need for extensive training and quality assurance.⁽⁶⁷⁾) In 2016, a WHO Expert Group⁽³⁰⁰⁾ recommended that the TB-LAMP assay “may be used as a replacement test for sputum smear microscopy for the diagnosis of PTB In adults with signs and symptoms consistent with TB (conditional recommendation, very low quality of evidence)” and that TB-LAMP ‘may be used as a follow-on test to smear microscopy in adults with signs and symptoms consistent with PTB, especially when further testing of sputum smear-negative specimens is necessary (conditional recommendation, very low quality of evidence).’ The Expert Group also remarked that due to the limited evidence, ‘it is unclear whether TB-LAMP has additional diagnostic value for the testing of PLHIV with signs and symptoms consistent with TB.’⁽³⁰⁰⁾

The Genedrive® MTB/RIF assay developed by Epistem is a rapid molecular TB test that utilises a simple paper-based DNA extraction method coupled with PCR amplification and detection on Epistem’s Genedrive® instrument [Figure 17].⁽³⁰¹⁾ A recent clinical evaluation⁽³⁰¹⁾ of the assay among 336 participants recruited from South Africa, Uganda and Brazil revealed that the overall sensitivity of the Genedrive® was 45.4% (95% CI [35.2, 55.8]) when using culture as the reference standard. In comparison, the sensitivities of the Xpert® and smear microscopy were 91.8% (95% CI [84.4, 96.4]), and 77.3% (95% CI [67.7, 85.2]), respectively.⁽³⁰¹⁾ The sensitivities of Genedrive® and Xpert® for the detection of smear-negative TB were 0% (95% CI [0, 15.4]) and 68.2% (95% CI [45.1, 86.1]), respectively.⁽³⁰¹⁾ These results contrast with the specifications claimed by the manufacturer of a sensitivity of 94% in smear-positive, culture-positive raw sputum specimens.⁽³⁰²⁾ The Genedrive® assay is undergoing further development, and the test developers are aiming to address the different issues highlighted by Shenai *et al.*⁽³⁰¹⁾



Figure 17. Key components of Epistem's Genedrive® *Mycobacterium tuberculosis* iD® system.⁽³⁰³⁾

Reproduced from Expert Review of Molecular Diagnostics; Vol. 12, Issue 7; Niemz A, Boyle DS; Nucleic acid testing for tuberculosis at the point-of-care in high burden countries; Pages 687-701; Copyright 2012, with permission from Taylor & Francis.

Key:

- (A) The Genedrive instrument, with ports at the front for the insertion of the test cartridge.
- (B) The Genedrive test cartridge, with three protruding clear reaction tubes that are inserted into the instrument port prior to test execution.
- (C) The user interface displays test results for *Mycobacterium tuberculosis* identification and RIF drug resistance testing.

TrueNat™ Uno, developed by Molbio Diagnostics Private Ltd (Goa, India), is a rapid, low-cost, semi-automated quantitative NAAT platform for TB diagnosis in remote healthcare settings.⁽³⁰³⁾ The assay is based on paramagnetic bead-based DNA extraction in a semi-automated fashion and is powered by either battery or line power.⁽³⁰³⁾ The DNA extract is manually transferred into a reaction cartridge containing lyophilised PCR reagents, which is inserted into the Truelab™ Uno machine for real-time PCR analysis

with two-channel excitation and photo-detection.⁽³⁰³⁾ The instrument houses an optical detection system, an internal rechargeable battery that can run the system for up to 8 hours, and an Android-type wireless device for data entry, platform control and processing, test analyses and result dissemination.⁽³⁰³⁾ As opposed to other PCR platforms, the reaction cartridge contains the thermocycling component that is controlled and powered via the instrument.⁽³⁰³⁾ Studies on the Truenat™ MTB test undertaken in India found sensitivity and specificity to be similar to that of the Xpert®MTB/RIF assay.^(304, 305)

A study from China using cross-priming amplification technology (CPA) [Ustar Biotechnologies, Hangzhou, China] using processed sputum reported sensitivity from smear- and liquid culture-positive specimens was 96.9%, and that from smear-negative and liquid culture-positive specimens was 87.5%.⁽²⁹⁸⁾ The specificity of CPA in culture-negative specimens was 98.8%.⁽²⁹⁸⁾ This technology amplifies a DNA target sequence at a constant temperature using multiple cross-linked primers (six to eight primers). The detection of amplified products is performed on a lateral flow strip housed in an enclosed, sealed plastic device to prevent the leakage of amplicons. Ou *et al.*⁽³⁰⁶⁾ reported that compared to solid culture, the sensitivity and specificity of the CPA test for MTB detection within this group was 84.1% (95% CI [79.5, 88.6]) and 97.8% (95% CI [97.1, 98.5]), respectively, and the sensitivity in smear-negative cases was 59.8% (95% CI [49.8, 69.8]).

There are several other PCR-based fully integrated *in vitro* diagnostic NAAT systems that are commercially available or in development, including the FilmArray® by BioFire Diagnostics, LLC (Salt Lake City, Utah, USA), cobas®Liat PCR System (Roche Diagnostics), and Enigma®ML assay (Enigma Diagnostics, Porton Down, Salisbury, UK).^(303, 307-309) Although there

are no TB assays currently offered on these platforms, TB NAAT can probably be accommodated.

5.2 Specimen types

5.2.1 Sputum

Microbiological diagnosis of PTB, particularly at primary care level, depends on obtaining an adequate expectorated sputum sample. Sputum is the specimen of choice for diagnosing pulmonary disease, but the mucoid and viscous nature of the sample makes it difficult to manipulate and often interferes with test performance.⁽¹³⁶⁾ The collection of good quality samples for proper diagnosis is difficult, and current sputum processing methods are crude.⁽¹⁷²⁾ Sample processing is usually necessary before diagnostic tests are applied, resulting in increasing complexity and cost.⁽¹³⁶⁾

Collection of sputum is difficult particularly in paediatric patients and sputum scarce adult patients. In up to a third of TB cases, an adequate biological sample is not readily available or has a very low concentration of TB bacilli rendering the sample smear-negative.⁽⁷⁶⁾ Lawn *et al.*⁽³¹⁰⁾ showed that of 427 HIV-infected adults, who were newly admitted to an acute medical ward in hospital, sputum (spot and/or induced samples) were obtainable from only 37% of patients in the first 24 hours of admission. The diagnosis of TB in HIV-infected patients remains particularly challenging due to the high rate of sputum smear-negative TB [at least 20% of all adults (up to 30-40% in HIV-infected patients) and EPTB disease].^(78, 311)

A recent systematic review⁽³¹²⁾ has found that sputum induction increases TB case detection and is useful for people who are negative on spontaneous smear microscopy or unable to expectorate spontaneously. From 23 studies which met the authors inclusion criteria, the overall success of sputum induction was high, ranging from 76.4% (95% CI [68.5, 83.2]) to 100% (95% CI [98.5, 100]), while adverse events associated with sputum induction were infrequent and mild.⁽³¹²⁾ It was well-tolerated by children and adults, irrespective of HIV status.⁽³¹²⁾ Sputum induction is relatively easy to perform at the primary health level.⁽³¹³⁾ Adequate biosafety training, personal protection measures (e.g. N95 masks) and adequate natural ventilation should be the norm in all sputum collection procedures.⁽³¹³⁾

5.2.2 Other specimens for the diagnosis of extrapulmonary TB

Extrapulmonary TB includes meningitis, genitourinary infection, pericarditis, lymphadenitis, pleurisy, peritonitis, musculoskeletal infection, and cutaneous TB.⁽³¹⁴⁾ Diagnosing EPTB remains even more challenging because clinical samples must be obtained from relatively inaccessible sites and are often paucibacillary, decreasing the sensitivity of diagnostic tests.^(77, 315) In addition, there are often inadequate specimen volumes, division of the sample for various diagnostic tests (histology/cytology, biochemical analysis, microbiology, and PCR), uneven distribution of bacteria within the specimen, and the lack of an efficient sample processing technique universally applicable to all types of extrapulmonary samples, all of which further reduce the sensitivity of diagnostic tests.⁽³¹⁵⁾ Invasive procedures place patients at increased risk of complications and result in higher costs.⁽³¹⁶⁾

5.2.2.1 Smear microscopy and mycobacterial culture

In EPTB, conventional AFB smears have low sensitivity (ranging 0% to 40%) and it requires a long time for *M. tuberculosis* to become evident during culture.^(77, 317) Although 40% of patients with EPTB may have concurrent PTB, the most widely available method of diagnosis, sputum smear microscopy, is of little diagnostic value for the remaining 60%.⁽³¹⁸⁾ Culture of body fluids or biopsy specimens is regarded as the reference standard for the diagnosis of EPTB.⁽³¹⁴⁾ The sensitivity of sputum culture varies in reported studies by site of EPTB: 28–50% for abdominal TB, 10–11% for tuberculous pericarditis, 24–29% for tuberculous meningitis, and 5–14% for tuberculous lymphadenitis.⁽³¹⁷⁾ Sensitivity of culture in specimen types other than sputum is variable (30–80%) with a TAT of 4–8 weeks.⁽³¹⁷⁾

5.2.2.2 Histopathological diagnosis

As a result of the poor sensitivity of smear microscopy, the diagnosis of EPTB mostly depends on histological evidence. For histopathological diagnosis, the presence of granulomas, caseation, and demonstration of AFB have been commonly used to define a positive test.⁽⁷⁷⁾ However, loss of host immune function can result in histopathologic findings demonstrating greater suppurative response and less well-formed granulomas.⁽³¹⁹⁾ Granulomas can also be seen in sarcoidosis, NTM disease, fungal infections, brucellosis, or syphilis, so cautious interpretation of these results is required.^(315, 320) In general, tissue biopsy yields positive culture results more often than fluid aspiration.⁽⁷⁷⁾

The selection of the diagnostic procedures depends on the organ involvement in EPTB.⁽⁷⁷⁾ Various methods that include needle biopsy; excision; endoscopy; laparoscopy; and biopsies under guidance of ultrasound, CT, or endoscopic ultrasound have been employed for diagnosis.⁽⁷⁷⁾ Although tissue biopsy is the most effective method of diagnosing EPTB, it is invasive and sometimes inaccessible.⁽⁷⁷⁾ Consequently, more easily accessible body fluids, such as pleural, peritoneal, and pericardial fluids, can often provide valuable diagnostic clues in EPTB patients.⁽⁷⁷⁾ A comprehensive evaluation of the additional value of extrapulmonary samples was made in a study⁽³²¹⁾ in South-East Asia in which patients (median CD4 T-cell count, 281 cells/ μ L) attending HIV clinics were intensively screened for TB, with collection of three sputum samples together with stool, urine, blood culture, and (when possible) lymph node aspirates. TB was diagnosed in 14% of patients overall; 86% of these diagnoses were made using sputum samples, and the additional yield of 14% of diagnoses were made using culture of non-pulmonary samples.⁽³²¹⁾ These authors also documented that lymph node aspiration provided the highest incremental yield of any of the non-pulmonary specimens tested for TB.⁽³²¹⁾

5.2.2.3 Adenosine deaminase

Measurement of adenosine deaminase (ADA) activity is one of the most studied and widely used biomarkers in body fluids for the diagnosis of EPTB. ADA is an enzyme involved in purine metabolism that is found in many tissues, particularly in T-lymphocytes from the lymphoid tissue.⁽³²²⁾ Activity of this enzyme increases in TB patients because of the stimulation of T-cell lymphocytes by mycobacterial antigens.⁽³²²⁾ It has been proposed to be a useful surrogate marker for TB in body fluids, such as pleural, pericardial, and peritoneal fluid, although possible false-negative and false-positive results may occur.^(323, 324) Raised ADA levels may be observed in a number of

conditions such as rheumatoid effusion, empyema due to other bacteria, mesothelioma, lung cancer, parapneumonic effusion, and haematological malignancies.^(323, 324) A systematic review⁽²²⁹⁾ of ADA by the NHS Health Technology Assessment Program which included 42 data sets from 36 published studies showed that the overall pooled analysis suggested that ADA tests have reasonably high sensitivity and specificity for pleural TB (Figure 18). However, there were many issues with studies included in the systematic review, such as none of the studies had used culture alone as a reference standard and none of the studies were judged to have chosen a representative patient spectrum.⁽²²⁹⁾ Two previous meta-analyses assessing ADA for detection of pleural infection also found ADA to perform well.^(322, 325) Therefore, in populations with a high prevalence of TB and clinical suspicion of TB effusion, elevated ADA level might be considered as a confirmatory test justifying treatment initiation in pleural, pericardial and peritoneal TB.⁽³²⁴⁾

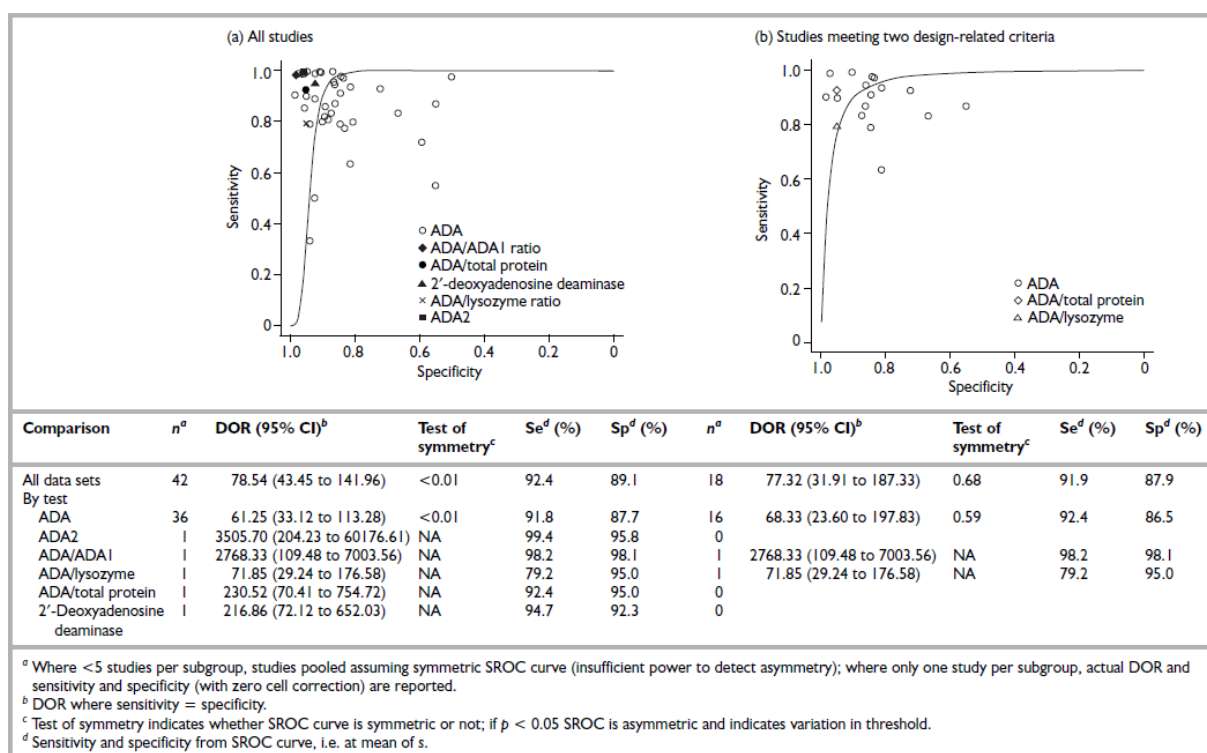


Figure 18. Adenosine deaminase evaluations in pleural TB-summary receiver operator curve (SROC) models and associated SROC curves from the systematic review performed by the NHS Health Technology Assessment Programme.⁽²²⁹⁾ Reproduced from the National Institute for Health Research Health Technology Assessment; Vol. 11, Issue 3; Dinnes J, Deeks J, Kunst H, Gibson A, Cummins E; Page 70; Copyright 2007 Queen's Printer and Controller of HMSO. (Licence available from: <http://www.nationalarchives.gov.uk/doc/non-commercial-government-licence/non-commercial-government-licence.htm>)

5.2.2.4 Mycobacteraemia

Mycobacteraemia is detected in many patients with HIV infection and active TB including children⁽⁸⁰⁾, and has also been noted as an important cause of fever among patients in hospital in geographic locations with high HIV infection rates.^(326, 327) Hence, blood culture is suggested as a tool to assist in the diagnosis of TB in HIV-infected patients especially those with disseminated disease^(72, 328), and in locations where atypical mycobacteria are

common.⁽³²⁹⁾ Several studies have shown that the detection of mycobacteraemia among people with HIV infection or AIDS varies widely between 19% and 96%.^(71, 72, 326, 330, 331) One study⁽³³¹⁾ found that identification of the presence of mycobacteraemia however did not improve patient outcomes. Moreover, mycobacterial blood culture is not cost-effective in resource-constrained settings.⁽³³²⁾

5.2.2.5 Nucleic acid amplification tests

The poor performance of conventional microbiological techniques in extrapulmonary specimens has stimulated the increased use of PCR tests.⁽³¹⁵⁾ However for the diagnosis of EPTB, NAATs generally do not perform as well as for sputum.^(229, 333-335) Accuracy varies considerably with sensitivity ranging from 27.3-100%, depending on the specimen type and test method.^(225, 229) Dinnes *et al.*⁽²²⁹⁾ who performed a systematic review on NAATs demonstrated a pooled sensitivity and specificity of 78.1% and 95.8%, respectively for miscellaneous extrapulmonary specimens (22 studies) and 72.6% and 93.7%, respectively for pleural TB (20 studies). For TB meningitis, pooled sensitivity was 58.6% and specificity was 96.2% (26 studies).⁽²²⁹⁾ Studies using the Xpert®MTB/RIF assay were not included in the review mentioned above.

In 2013, the WHO Expert Group reviewed the performance of the Xpert®MTB/RIF assay to diagnose EPTB in adults and children.⁽³³⁶⁾ The group reviewed 15 published and seven unpublished studies involving 5,922 specimens.⁽³³⁶⁾ The results of their meta-analysis are shown in Table 4. The Expert Group recommended the Xpert®MTB/RIF assay should be “used in preference to conventional microscopy and culture as the initial diagnostic test for cerebrospinal fluid specimens from patients suspected of having TB

meningitis (strong recommendation; very low quality evidence)” and that the Xpert®MTB/RIF assay may be used as a “replacement test for usual practice (including conventional microscopy, culture or histopathology) for testing specific non-respiratory specimens (lymph nodes and other tissues) from patients suspected of having EPTB (conditional recommendation, very low quality evidence).”⁽³³⁶⁾

Table 4. Meta-analysis of the sensitivity and specificity of the Xpert®MTB/RIF assay in diagnosing extrapulmonary tuberculosis against culture as a reference standard as well as a composite reference standard, by type of extrapulmonary specimen.

Specimen type	Comparison (No. of studies, No. of samples)	Median (%) pooled sensitivity (pooled 95% CrI)	Median (%) pooled specificity (pooled 95% CrI)
Lymph node tissue and aspirate	Xpert MTB/RIF compared against culture (14 studies, 849 samples)	84.9 (72–92)	92.5 (80–97)
	Xpert MTB/RIF compared against a composite reference standard (5 studies, 1 unpublished)	83.7 (74–90)	99.2 (88–100)
Cerebrospinal fluid	Xpert MTB/RIF compared against culture (16 studies, 709 samples)	79.5 (62–90)	98.6 (96–100)
	Xpert MTB/RIF compared against a composite reference standard (6 studies, 512 samples)	55.5 (51–81)	98.8 (95–100)
Pleural fluid	Xpert MTB/RIF compared against culture (17 studies, 1385 samples)	43.7 (25–65)	98.1 (95–99)
	Xpert MTB/RIF compared against a composite reference standard (7 studies, 698 samples)	17 (8–34)	99.9 (94–100)
Gastric lavage and aspirate	Xpert MTB/RIF compared against culture (12 studies, 1258 samples)	83.8 (66–93)	98.1 (92–100)
Other tissue samples	Xpert MTB/RIF compared against culture (12 studies, 699 samples)	81.2 (68–90)	98.1 (87–100)

Reproduced from the World Health Organisation’s “Automated real-time nucleic acid amplification technology for rapid and simultaneous detection of tuberculosis and rifampicin resistance: Xpert MTB/RIF assay for the diagnosis of pulmonary and extrapulmonary TB in adults and children. Policy Update.”⁽³³⁰⁾

Key: CrI-credible interval (Bayesian equivalent of confidence interval).

5.3 The way forward...

Rapid technological advances in the past decade have led to a revolution in TB diagnosis. There has been major progress in the development of new diagnostic tests for TB.^(90, 144) As mentioned previously, old technologies have been reviewed and improved and new technologies have been developed, evaluated and implemented.⁽⁹⁰⁾ There has also been progress in TB control programmes, introduction and rollout of tests, and improvements in translational research whereby the impact of the introduction of such tests has been evaluated.⁽¹⁴⁴⁾ The WHO issued ten policy statements between 2007 and 2012 regarding TB diagnosis and diagnostic methods, which highlights the massive progress that has been made.^(67, 90)

Although the past five years have seen an unprecedented activity in the development of a range of new TB diagnostic tests based on culture, molecular, and non-molecular methods, a major concern is that not all of these marketed tests have been assessed rigorously for diagnostic accuracy, robustness under operational conditions in the field, cost-effectiveness, or practical usefulness.⁽³³⁷⁾ In 2013, no data on the performance characteristics of any new diagnostic technology was submitted to the WHO for the systematic evaluation process used in the formulation of policy guidance.

Despite the advances, it is estimated that one third of all TB cases are missed.⁽²⁶⁾ There remains an urgent need to develop rapid, simple, and accurate TB diagnostic tools. Accurate and rapid detection of TB is crucial for not only improving patient outcomes but decreasing TB transmission. Novel tests that reach “the missing three million patients” and curb the epidemic of drug-resistant TB are needed.⁽⁹⁶⁾ Although such tests are under development and validation, policy and clinical practice should also be modified to

improve the diagnosis and management of TB, particularly smear-negative TB in HIV-infected patients, which is associated with poorer outcomes.⁽⁷²⁾

5.3.1 Potential impact of a new test

There are two opportunities where intervention with improved diagnostic tools might aid case detection and reduce transmission: firstly, in active screening to detect new cases in the community before patients become symptomatic, and secondly, to improve the investigation of symptomatic patients presenting at the clinic.⁽⁸⁹⁾

The overall impact of any new diagnostic tests for active TB disease or latent *M. tuberculosis* infection will depend on the extent of their uptake into national TB programs, affordability both from the patient and health system perspective, the quality and durability of the diagnostic devices, and access to appropriate treatment following diagnosis.⁽³³⁸⁻³⁴⁰⁾ The need to increase research and development into POC tests for TB has received increasing attention in recent years, but there is still a lack of a focused and strategic approach and insufficient integration between areas of biological discovery and test development and the establishment of well-characterised specimen repositories for initial test evaluation.^(51, 341) Post 2015 targets are unlikely to be met without the use of novel diagnostics that can be integrated into affordable diagnostic algorithms close to patient care.⁽⁹⁶⁾ A rapid and universally accessible test that is not affected by HIV status, with a sensitivity of 85% and a specificity of 97%, has the potential to save 392,000 adjusted lives annually, or 22% of the global TB deaths.⁽⁹⁷⁾

5.3.2 The 'ideal' test

What is required is a rapid, affordable, accessible, simple, user-friendly POC assay that will obviate the need for return visits for test results; promote same day treatment initiation, and reduce loss to follow-up.⁽⁵¹⁾ The ideal test would be highly sensitive (>90%) and specific (>95%) compared to culture.⁽³⁴²⁾ The test should preferably be performed on easily obtained specimen types, require minimal processing or technical and training requirements.^(342, 343) In addition, the test should have a long shelf-life, maintain stability in a range of temperatures and humidity levels, and require minimal maintenance whilst being cost-effective.^(342, 343) The ideal test would be environmentally friendly, and should work off battery or solar power as well.⁽³⁴²⁾ The test should be able to be performed at various levels of healthcare (from primary health clinic to quaternary hospital level). The device should preferably have a high throughput; the ability to be used on various biological specimens, and be carried out by any healthcare worker with minimal training. The test result should be easy to read, and should also provide some drug susceptibility results as well. The ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end users) criteria, outlined by the WHO⁽³⁴⁴⁾, describes the ideal characteristics for evaluating POC devices for resource-limited environments.^(179, 345)

5.3.3 Specimen type and its influence on test specifications

The success of any new diagnostic test will depend on the ability to obtain good quality material from the site of disease, which is often not trivial in the environments where POC diagnostics are most needed.⁽³⁴⁶⁾ Identification of samples other than sputum, such as blood, urine, breath, lung or gastric

lavage fluid, biopsies, aspirates or effusions, is critical for improving access to diagnosis.⁽³⁴⁶⁾

In developing novel tests for paediatric and EPTB, one approach could be an “optimised” Xpert® or other NAAT capable of detecting *M. tuberculosis* with higher sensitivity in specimens other than sputum (e.g., tissue) through improved sample processing and DNA extraction. Another approach targeting the diagnosis of PTB in children and sputum scarce adults might be an improved non-sputum-based assay using more easily accessible specimens. Therefore, there is a great interest in using bodily fluids and solids that can easily be obtained, such as breath, urine and stool, for the diagnosis of PTB and/or disseminated TB.⁽³⁴²⁾

Urine and stool *M. tuberculosis* DNA detection methods offer some promise on this front.⁽³⁴⁷⁻³⁵⁰⁾ Urine testing is particularly attractive as the specimen is fairly easy to collect, process and store. It is also less of a biological hazard to healthcare workers. Transrenal DNA, or small fragments of microbial DNA (<200 base pairs) filtered through the kidneys, can be detected using NAATs for diagnosis of both PTB and EPTB.⁽³⁵¹⁾ Of seven studies^(348, 351-356) evaluating the diagnostic accuracy of transrenal DNA detection for nonrenal or urethral TB, four compared test performance in HIV-infected and HIV-uninfected patients.^(352, 354-356) Although specimen processing varied from study to study, all four studies^(352-354, 356) found higher sensitivity among HIV-infected subjects (16-64%) compared with HIV-uninfected individuals (6-38%).⁽³⁵⁷⁾ If consistency in test performance can be achieved by optimising and standardising specimen processing, transrenal DNA detection may be a valuable tool for diagnosing TB in the HIV-infected population. A few studies have examined the use of urine specimens from HIV-infected patients clinically suspected of having TB in the Xpert®MTB/RIF platform.^(310, 358, 359) Peter *et al.*⁽³⁵⁸⁾ demonstrated that the sensitivity (48%) of

urine Xpert®MTB/RIF was comparable to the sensitivity (52%) of smear microscopy in this study population. These authors showed that the sensitivity was improved (54%) in those with CD4 T-cell counts <200 cells/μL compared with those with CD4 T-cell counts >200 cells/μL (31%).⁽³⁵⁸⁾ Similarly, Lawn *et al.*⁽³⁵⁹⁾ demonstrated that the ability of the Xpert®MTB/RIF to detect TB in urine was lower, but the same trend was observed as in the previously mentioned study (CD4 T-cell count <50 cells/μL: sensitivity 44%; CD4 T-cell count 50-150 cells/μL: sensitivity 25%; and CD4 T-cell count >150 cells/μL: sensitivity 3%). In a more recent study, Lawn *et al.*⁽³¹⁰⁾ showed that of 427 HIV-infected adults acutely admitted to hospital, the proportions of total TB cases ($n=139$) that were diagnosed by Xpert® testing of sputum, urine, or both sputum and urine combined, within the first 24 hours were 39/139 (28.1%), 89/139 (64.0%) and 108/139 (77.7%) cases, respectively ($p<0.001$). Although the ability to detect TB in urine using the Xpert®MTB/RIF platform is currently suboptimal, it may be of value in HIV-infected patients with advanced immunosuppression who are unable to produce sputum.⁽⁶⁷⁾

Cordova *et al.*⁽³⁶⁰⁾ hypothesized that *M. tuberculosis* DNA could be detected in stool from adults with PTB. For newly diagnosed PTB patients, stool *IS6110* PCR had 86% sensitivity and 100% specificity compared with results obtained by sputum culture, and stool PCR had similar sensitivities for HIV-infected and HIV-uninfected patients ($p=0.3$).⁽³⁶⁰⁾ A study⁽³⁶¹⁾ of 1,693 HIV-infected patients in South-East Asia revealed that 44% of those with culture-confirmed TB also had a positive stool culture, and a small paediatric study⁽³⁶²⁾ using Xpert®MTB/RIF on stool and gastric aspirate samples detected TB in three of four (75%) children with intrathoracic TB and positive gastric aspirate cultures and 3 of 6 (50%) children with *M. tuberculosis* cultured from any site.

5.3.4 POC testing

In the last 20 years, the availability and use of POC tests have greatly increased and expanded to all fields of medicine, so that a significant proportion of laboratory testing is currently conducted at the point of care.⁽³⁶³⁻³⁶⁵⁾ In the setting of infectious diseases, most existing POC tests consist of immunoassays, namely agglutination, immunochromatographic, and immunofiltration tests.⁽³⁶⁶⁾ POC tests based on nucleic acid detection are already available for some pathogens, such as *Clostridium difficile*, *Streptococcus pyogenes*, HIV and influenza, and represent a major advance. The decrease in analytical time in comparison with standard microbiological procedures offers potentially substantial benefits for the management of infectious diseases.⁽³⁶⁷⁾

Several definitions of a POC test exist, based on a geographical, functional, technological, or operational context.^(368, 369) An early definition was “*a medical test that is conducted at or near the site of patient care*”.⁽³⁷⁰⁾ Another definition was “*any test that is performed at the time at which the test result enables a clinical decision to be made and an action taken that leads to an improved health outcome*”.⁽³⁶³⁾ More recently, experts on HIV and TB diagnostic testing defined a POC test as “*a diagnostic test that is performed near the patient or treatment facility, has a fast turnaround time, and may lead to a change in patient management*”.⁽³⁷¹⁾

Point-of-care tests have the potential to improve the management of TB, especially in resource-limited settings where healthcare infrastructure is weak, and access to quality and timely medical care is a challenge.^(179, 369, 372) These tests offer rapid results, allowing for timely initiation of appropriate therapy, and/or facilitation of linkages to care and referral. Most

importantly, POC tests can be simple enough to be used at the primary care level and in remote settings with no laboratory infrastructure.⁽³⁶⁹⁾ Diagnostic POC testing is intended to minimise the time to obtain a test result, thereby allowing clinicians and patients to make an expeditious clinical decision.

As POC testing expands into low-income settings, conducting appropriate evaluations of accuracy is paramount.⁽³⁷³⁾ A fundamental criterion for success of any diagnostic POC test is its accuracy and reliability.⁽³⁷⁴⁾ However, test results from a controlled laboratory environment may not be adequate or appropriate, since the test performance could significantly differ when a POC test is operated at the clinical point-of-care.⁽³⁷⁵⁾ The accuracy of any POC test may be reduced when operated in a clinical setting than in a laboratory environment.⁽²²⁹⁾ Analyses of diagnostic POC tests that rely on laboratory-based evaluations may give an inaccurate representation of a test's performance characteristics in a real world setting.⁽³⁷³⁾ The most appropriate measure of accuracy will be generated when a POC test is evaluated in the setting and location in which it will be used (i.e., at the clinical point-of-care).⁽³⁷³⁾

After assessing diagnostic accuracy of a POC test when used at the clinical point-of-care, the next step would be evaluating the clinical impact of POC testing on patient-centred outcomes. A POC test with seemingly inferior diagnostic accuracy when compared to a reference laboratory test may still be a valuable test that warrants clinical evaluation.⁽³⁷³⁾ Since the goal of POC testing is to expedite a clinical decision to improve patient outcomes, a POC test that is neither as sensitive nor as specific as a reference standard test may still have important clinical and public health benefits, if clinicians and patients can act on the results more frequently or quickly.⁽³⁷³⁾

Implementation of a NAAT globally is estimated to reduce TB incidence by 28% overall by 2050, whilst a dipstick test for antigens or antibodies reduces incidence by 42% at 2050.⁽³⁷⁶⁾ Denkinger *et al.*⁽²⁸⁶⁾ reported that POC sputum NAATs, though only able to diagnose PTB, reduced projected paediatric TB deaths by 13.3% (95% uncertainty range (UR) [4.6, 15.7]) and adult EPTB deaths by 8.4% (95% UR [2.0, 9.3]) simply by averting transmission of disease.

Modelling work has shown that a test with a performance better than smear (detection of 50% of smear-negatives), yet inferior to Xpert®, if employed at microscopy centres and combined with early treatment, would result in a reduction in transmission of TB over deployment of Xpert® at district level.^(96, 287) Moreover, such a test would increase the number of patients diagnosed with TB, thus potentially reducing transmission and morbidity by facilitating earlier diagnosis and treatment. If the test had a sensitivity better than the Xpert® (diagnostic sensitivity of >95% in comparison to culture; analytical sensitivity of less than 4.5 genome equivalents/reaction and <10² CFU/assay on one sample), it possibly will reduce empiric therapy and thus overtreatment.⁽⁹⁶⁾ Furthermore, if the replacement test could be used for monitoring purposes, it would be able to replace smear microscopy and is more likely to be adopted by TB programmes.⁽⁹⁶⁾

5.3.4.1 Clinical placement of POC tests

Diagnostic tests have the potential to improve patient care when the results are available to clinicians in a meaningful time frame and are reliable enough to influence treatment decisions.⁽³⁴³⁾

Denkinger *et al.*⁽³⁷⁷⁾ through interviews with representatives from national TB programmes, clinical experts from industrialised, middle- and low-income countries, researchers, and clinical laboratory experts, compiled a “wish-list” defining the most important diagnostic needs for TB. This “wish-list” included a triage and screening test, tests for patients with difficult to diagnose TB (children, HIV-infected patients and EPTB), a simple non-sputum based biomarker test for diagnosis of active TB, a molecular smear-replacement test based at the microscopy centre level, DST that could be done in decentralised or centralised settings, a biomarker test for diagnosis of latent TB infection that predicts progression to active TB, and a test for treatment monitoring.⁽³⁷⁷⁾ From this list, the authors ultimately identified the following tests as key priorities which would have the most impact on incidence and morbidity reduction:

1. “A rapid sputum-based test as a replacement for smear microscopy with or without DST (*‘smear replacement test’*);
2. a rapid non-sputum-based test capable of detecting all forms of TB via the identification of characteristic biomarkers or biosignatures (*‘non-sputum-based biomarker’*) ideally suitable for use at levels below microscopy centres;
3. a simple, low cost triage test for first contact healthcare providers (community healthcare workers) as a rule-out test (*‘triage test’*); and
4. a *rapid drug susceptibility test* for use at the microscopy centre level.”⁽³⁷⁷⁾

At a consensus meeting in 2014, the WHO⁽³⁷⁸⁾ proposed four highest priority testing indications (as above) as well as test target product profiles (Table 5).

Table 5. Minimum target product profiles for high priority new TB diagnostics. Reproduced from the World Health Organisation’s “High priority target product profiles for new tuberculosis diagnostics: report of a consensus meeting.”⁽³⁷⁸⁾

Test	Rapid, biomarker-based non-sputum-based test for detecting TB	Community-based triage or referral test to identify people suspected of having TB	Rapid sputum-based test for detecting TB at the microscopy-centre level of the healthcare system	Next-generation drug-susceptibility test
Goal and potential market	Test that can diagnose PTB and ideally also EPTB using non-sputum samples for the purpose of initiating TB treatment during the same clinical encounter or on the same day	Test used during a patient’s first encounter with the healthcare system to identify patients with any symptoms or risk factors for active PTB	A sputum-based test to detect PTB to be used at the level of a microscopy centre to support initiation of TB therapy during the same clinical encounter or on the same day	Diagnosis of TB disease and detection of drug resistance to inform decision-making about the optimal first-line regimen
Drug-susceptibility testing	None	None	None	
Target population	Adults and children (including HIV-infected) who are suspected of having active PTB or EPTB in countries with a medium-high prevalence of TB as defined by WHO	Adults and children suspected of having active, PTB in countries with a medium-high prevalence of TB as defined by WHO	All patients suspected of having PTB and able to produce sputum, in countries with a medium-high prevalence of TB as defined by WHO	All patients suspected of having TB, with a special focus on those at high risk of morbidity and mortality from drug-resistant TB
Target user	Trained microscopy technicians	Staff trained to the level of auxiliary nurses	Healthcare workers who have had a minimum of training	Healthcare workers with training necessary for performing smear microscopy
Setting	Primary healthcare clinics, peripheral level microscopy centres or higher levels of the healthcare system	Health posts and primary-care clinics or higher levels of the healthcare system	Microscopy-centre level	Microscopy-centre level or higher levels of the healthcare system
Price	< US\$ 6.00	< US\$ 2.00	<US\$ 6.00	< US\$ 10.00
Diagnostic sensitivity for TB detection	PTB in adults: Sensitivity should be ≥ 65%, but should >98% among patients with smear-positive culture-positive TB. For children: No lower range of sensitivity was defined.	Overall sensitivity should be > 90% when compared with the confirmatory test for PTB	Sensitivity ≥ 80% for a single test when compared with culture; sensitivity for smear-negative TB should be > 60%, and for smear-positive it should be 99%	Sensitivity should be > 80% for a single test when compared with 2 liquid cultures; for smear-negative TB it should be > 60%; for smear-positive TB it should be 99%
Diagnostic specificity for TB detection	The test should have 98% specificity when compared against a microbiological reference standard	Overall specificity should be > 70% when compared with the confirmatory test	≥ 98% specificity compared with culture	Specificity should be > 98% for a single test when compared with culture
Sample type	Non-sputum-based samples (such as urine, blood, oral mucosal transudates, saliva, exhaled air)	Sputum; a non-sputum sample type would be preferred	Unprocessed sputum	Unprocessed sputum
Sample volume	Urine: < 10 mL; Finger-stick blood: < 25 µL; Saliva or transudates: <0.2 mL	Urine: < 10 mL; Finger-stick blood: < 25 µL; Saliva or transudates: <0.2 mL; Sputum: < 0.1 mL	0.5–5 mL	< 0.5–2 mL
Time to result	< 1 hour including time spent preparing the sample	< 30 minutes including time spent preparing the sample	< 2 hours including time spent preparing the sample	< 2 hours for DST alone
Biosafety	No need for a biosafety cabinet	No need for a biosafety cabinet	Same as smear microscopy	Should have the same requirements as the Xpert®MTB/RIF assay
Instrument	Preferably the test would not require an instrument; if an instrument is required, it should be small, portable or hand-held and weigh < 1 kg; it should be able to operate on battery or solar power in places where the power supply may be interrupted	A small, portable or hand-held device weighing < 1 kg	Up to 2 instruments that are independent of each other	Up to 2 instruments that are independent of each other
Power requirements	Optional battery or solar-powered operation	Optional battery or solar-powered operation	Battery operated with recharging capability and a circuit protector	Capable of running on standard electricity plus an uninterrupted power supply unit to enable a cycle to be completed in case of a power outage

5.3.4.1.1 Triage test

A triage test is a test that is used by first contact healthcare providers to identify those who need further diagnostic testing.⁽³⁷⁸⁾ Ideally, this test needs to be a simple, low-cost test with high sensitivity. Such a test should rule out TB when the result is negative.⁽³⁷⁷⁾ Individuals with a positive result are then directed to further evaluation with a confirmatory test, e.g., Xpert®MTB/RIF assay. The sensitivity of a triage test should ideally be as good as that of the confirmatory test (>95% of confirmatory test), otherwise patients could be missed by the test and the strategy of testing all patients with the confirmatory test would theoretically result in a higher case notification rate.⁽³⁷⁷⁾ However, if a triage test is done at lower levels of care and is easier to perform; conceivably more people suspected of having TB will be tested. Consequently, the test might increase the number of TB patients identified even if its sensitivity is lower than that of the confirmatory test.⁽³⁷⁷⁾ From the 2014 consensus meeting, the WHO experts recommended that the overall sensitivity should be >90% when compared with the confirmatory test for PTB.⁽³⁷⁸⁾

For successful implementation at the community level, a triage test should ideally use an easily accessible sample (e.g., urine, finger-stick blood, breath).⁽³⁷⁸⁾ The test should optimally be device-free or if a device is needed, it should at least be battery-operated.^(295, 377, 378) The ideal time-to-result (including sample preparation and processing time) has not been studied; however, a rapid test is more likely to be integrated within workflow and result in same visit decision-making.^(95, 96)

5.3.4.1.2 Sputum-based replacement for smear microscopy

A POC sputum test to replace smear microscopy would be feasible at the microscopy centre level and would support the prompt initiation of therapy, thereby reducing TB transmission and morbidity, while leveraging existing infrastructure.⁽¹⁴⁴⁾ The ideal sputum replacement test should have a fast TAT to rapidly inform a treatment decision at the first visit.^(377, 379, 380) Botha *et al.*⁽³⁸¹⁾ reported in their study that of 2,037 participants suspected of having TB with at least two smear microscopy results, 64 (17%) of 367 participants with two positive smears did not start treatment due to various diagnostic errors in sputum sample collection, transport to the laboratory and receipt of results.

The test should be robust, require simple sample preparation, minimal sample handling and minimal operation requirements.^(95, 96) Such a test must be run on solar power or batteries, handle extremes of temperature and environments (e.g., high humidity, dusty environments). Only minimal expertise and tools should be required for maintenance and repair of test equipment given the difficulty of service visits to peripheral microscopy centres.^(95, 377)

A highly sensitive test based on a biological sample other than sputum (such as urine, blood, saliva, or exhaled air) suitable for implementation at lower levels of care would help shorten the delay before diagnosis and enable early treatment (and thus reduce morbidity, mortality and transmission).⁽²⁸⁶⁾ A non-sputum-based sample could also enable the diagnosis of EPTB and TB in children as well as the diagnosis in patients presenting in an earlier stage of the disease (e.g., patients who do not have a productive cough).⁽³⁷⁷⁾

5.3.4.2 Novel POC tests in development/evaluation

5.3.4.2.1 Reporter enzymes

Reporter enzyme fluorescence technology detects bacterial enzyme products at a thousandfold lower protein levels than detection of fluorescent proteins.⁽¹⁶⁴⁾ *Mycobacterium tuberculosis* expresses a beta(β)-lactamase enzyme which has a unique substrate binding site which permits binding of specific substrates not catalysed by β -lactamases produced by other bacteria.⁽¹⁶⁴⁾ A POC test based on this principle called the GBD TB REaD™ assay is being co-developed by Global BioDiagnostics (Temple, Texas, USA) with the Foundation for Innovative New Diagnostics. The test is a fluorimetric substrate-based assay for detection of *M. tuberculosis* β -lactamase (TB-*blaC*) in sputum samples.⁽¹⁶⁴⁾ The test is expected to have diagnostic accuracy comparable to culture.⁽¹⁶⁴⁾

5.3.4.2.2 Volatile organic compound detection

5.3.4.2.2.1 Breath

More than 3,500 volatile organic compounds (VOCs) have been documented to date.⁽³⁸²⁾ Biomarkers identified from exhaled breath include nitric oxide; inflammatory indicators related to oxidative stress, such as hydrogen peroxide and isoprostane; other nitrogen oxides; metabolites of arachidonic acid and cytokines; and bacterial products.⁽¹⁶⁴⁾ Progress has been made in identifying patterns that indicate active PTB from a patient's breath.^(383, 384) The major drawback of these tests has been the specificity and the limit of

detections of the VOCs [which are usually found in picomolar (part per trillion) concentrations]. A more detailed description of the various breath tests follows in chapter two.

5.3.4.2.2.2 Urine

Banday *et al.*⁽³⁸⁵⁾ have suggested that patients with TB can be distinguished from healthy controls by a rise in *o*-xylene and isopropyl acetate, and reduced levels of 3-pentanol, dimethylstyrene, and cymol in their urines. Further studies are required to confirm these findings.

5.3.4.2.3 Lipoarabinomannan

Lipoarabinomannan (LAM), a cell wall component of *M. tuberculosis*, has been shown to have some diagnostic merit in HIV/TB co-infected patients, particularly hospitalised patients with low CD4 T-cell counts.⁽³⁸⁶⁾ The test can be performed on urine in either an ELISA or lateral flow format. A detailed discussion of this test follows in chapter three.

5.3.4.2.4 Bioaerosol mass spectrometry (BAMS)

Aerosol-based novel diagnostics for TB are also being explored.⁽³⁸⁷⁾ Investigators at Livermore Instruments are evaluating a single-particle laser desorption/ionisation time-of-flight mass spectrometry tool for diagnosis of TB.^(387, 388) Pilot preclinical data suggest that *M. tuberculosis* particles could be identified in bioaerosol generated by the cough of an infectious TB patient.^(387, 389) However, BAMS systems are large and costly. With additional

engineering, this reagent-free, rapid (<2 minutes per patient) platform could significantly transform current approaches to screening for TB.⁽³⁸⁸⁾

5.3.4.2.5 MDR XDR-TB Colour Test

The MDR XDR-TB Colour Test is a rapid colourimetric DST. It combines the thin-layer agar technique with a simple colour-coded quadrant format, selective medium to reduce contamination, and colourimetric indication of bacterial growth to simplify interpretation.⁽³⁹⁰⁾ DST patterns for INH, RIF and ciprofloxacin can be determined.⁽³⁹⁰⁾ The test entails incubating a MDR XDR-TB Colour Test plate after inoculation with a patient's sputum specimen, and monitoring for colour change whilst being incubated.⁽³⁹¹⁾ Areas of colour change are examined with a conventional microscope to confirm growth of *M. tuberculosis*.⁽³⁹¹⁾ The MDR XDR-TB colour test shows utility as a screen for drug-resistant TB amongst newly diagnosed patients as well as testing symptomatic patients to approximately double the sensitivity of sputum-smear microscopy.⁽³⁹¹⁾

Toit *et al.*⁽³⁹⁰⁾ who evaluated the test using 201 archived *M. tuberculosis* isolates reported that the MDR XDR-TB Colour Test detected drug resistance with 98% sensitivity for INH, RIF and ciprofloxacin, and 99% for MDR-TB. Specificities for INH, RIF, ciprofloxacin and MDR-TB were 100% (95% CI [82, 100]), 88% (95% CI [69, 97]), 91% (95% CI [83, 96]), and 90% (95% CI [74, 98]), respectively.⁽³⁹⁰⁾ Agreement between the Colour Test and BACTEC MGIT 960 for RIF, INH, ciprofloxacin and MDR-TB were 98%, 96%, 94% and 97% respectively.⁽³⁹⁰⁾

6. CONCLUSION

The Global Plan to Stop TB partnership goals are to eliminate TB as a public health problem by 2050 (defined as <1 case per million globally).⁽²⁰⁴⁾ No single intervention will achieve this, but modelling studies have shown that new diagnostic tests could make a substantial impact.^(97, 376) However, the impact of even the most promising new diagnostic test will be highly dependent upon the system within which it is used and, more specifically, whether it reaches the patients who may benefit from it. A crucial step toward accomplishing these goals is improving diagnostic accuracy.

The goals for new diagnostics in the Global Plan to Stop TB 2011-2015⁽²⁰⁴⁾ are to have:

- 1) “a simple, rapid, and affordable test for use at peripheral health centres;
- 2) a test for MDR-TB at peripheral health centres; and
- 3) a test for latent TB infection that can identify people at high risk for disease progression. “

Although the Xpert®MTB/RIF assay and LPAs mark a significant step toward accomplishing the first two goals, these molecular methods have not been able to match the sensitivity of liquid culture systems, which remain the reference standard for diagnosing TB in HIV-infected patients (as well as in HIV-negative individuals). With limited finances, priority must be given to the development of technologies that will reach those not being served by current diagnostic provision.

This PhD constitutes a collection of diagnostic research trials of tests that potentially fall into the categories as described by Denkinger *et al.*⁽³⁷⁷⁾ in the discussion above. The following potential POC tests were studied in a high

prevalence setting of TB and HIV co-infection in a lower middle-income setting:

- an evaluation of a chemiresistive sensor ('TB breath test') which could be utilised as a smear replacement test as well as a non-sputum-based biomarker test;
- an evaluation of the urine LAM lateral flow test which also potentially fills the niche of a smear replacement test as well as a non-sputum-based biomarker in addition to a hospital triage test; and
- an evaluation of the use of the GeneXpert®MTB/RIF assay on blood specimens from HIV-infected patients which potentially could be used as a non-sputum-based smear replacement test in addition to a potential hospital triage test.

The final chapter focuses on a redesign of the sputum collection device in an effort to improve the use of the device for easier collection of sputum, reduction of the need for biosafety equipment at the peripheral microscopy centre as well as permitting integration into a fully automated molecular testing system.

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CHAPTER TWO: A FIELD EVALUATION OF A CHEMIRESTISTIVE SENSOR ARRAY FOR THE RAPID DIAGNOSIS OF PULMONARY TUBERCULOSIS

1. INTRODUCTION

The global pandemic of tuberculosis (TB) has highlighted the need for new diagnostic screening tests that are rapid and accurate.⁽¹⁾ Sputum smear microscopy forms the basis of diagnosis in resource-poor countries with a high TB burden, but the low sensitivity of this test results in patients with smear-negative, but culture-positive pulmonary tuberculosis (PTB) remaining undetected in current healthcare systems.⁽²⁾ The detection of volatile organic compounds (VOCs) produced by *Mycobacterium tuberculosis*⁽³⁾, directly from the breath of a patient suspected of having active disease, is an attractive alternative diagnostic technique. Such a test could be performed at the point-of-care (POC), is rapid, cost-effective, and non-invasive. It would also limit the exposure of healthcare and laboratory workers to potentially infectious sputum. An additional advantage is that the test could be performed in patients in whom sputum cannot be expectorated, are too ill for sputum induction procedures, or in paediatric patients in whom sputum specimens are difficult to obtain.

1.1 Olfactory diagnosis in medicine and other applications

In ancient times, physicians relied on their senses of colour, smell and taste to detect biological markers.⁽⁴⁾ Certain breath aromas are highly characteristic of specific diseases, for example, starvation victims with ketoacidosis generally have a sweet, acetone-like breath due to elevated levels of ketones in the breath whereas cyanide ingestion is indicated by a bitter-almond breath odour.^(5, 6) Similarly, bacteria are known to produce characteristic odours due to VOCs which are produced as part of distinct metabolic pathways.⁽⁷⁾ The premise of a breath test is that bacteria produce unique combinations of VOCs which can be used to identify the genus and species of the bacterium.⁽⁸⁻¹¹⁾ This principle of VOC identification from a patient's breath has been demonstrated in preliminary studies to detect cases of lung cancer⁽¹²⁻¹⁴⁾ and pneumonia⁽¹⁵⁾ using diagnostic breath signatures detected by breath sensors or 'electronic noses'.

The ability of humans to diagnose disease by olfaction has rarely been the subject of studies; however, there are several diagnostic studies suggesting that scent detection by animals is similar or superior to standard diagnostics for certain diseases, such as the use of dogs for the detection of lung cancer.⁽¹⁶⁾ Amongst mammalian species, rodents have been found to have the highest number of olfactory receptor genes.^(17, 18) Recent studies have shown that TB can be detected by trained African pouch rats (*Cricetomys* sp.) due to their ability to detect a blend of *M. tuberculosis*-specific volatile organic compounds from the sputum of infected patients.^(19, 20) The rats have been reported to detect TB with a sensitivity of greater than 86% and a specificity of more than 91%.⁽²⁰⁻²³⁾ It takes six to nine months to train a rat to detect TB and the cost of training is approximately 6,000 euro.⁽²⁰⁾ Once fully trained, a

rat can screen 140 sputum samples in 40 minutes.⁽²⁰⁾ A recent study in Tanzania⁽²⁴⁾ showed that of 109 (23.2%) culture-positive participants and 128 (27.3%) non-TB controls, at an optimal threshold defined at ≥ 2 indications by one or more of seven rats of either of two sputum samples, sensitivity and specificity was 56.9% [95% CI 47.0–66.3] and 80.5% [95% CI 72.5–86.9], respectively. Positive and negative predictive values were 71.3% (95% CI 60.6–80.5) and 68.7% (95% CI 60.6–76.0), respectively, with an accuracy for TB diagnosis of 69.6%.⁽²⁴⁾ Based on these results, whilst the rats show potential for TB detection, they currently do not meet the requirements for high-priority, rapid sputum-based TB diagnostics as defined by the WHO.⁽²⁴⁾ In addition, it is unlikely that laboratories in TB-endemic areas that handle large volumes of TB specimens will have the capacity to house and care for the large numbers of these rodents necessary to handle the heavy workload.

The earliest artificial olfaction device can be traced back to 1961 when Moncrieff⁽²⁵⁾ proposed a mechanical artificial nose.⁽²⁶⁾ The concept of an electronic nose using a chemical sensor array system for odour classification was proposed by Persaud in the 1980's.^(26, 27) Since then advances in sensor technology, electronics, biochemistry and artificial intelligence have accelerated the development of devices capable of measuring and characterising VOCs released from a multitude of sources for numerous applications.⁽⁵⁾ Hundreds of different prototypes have been developed to discriminate complex vapour mixtures containing many different types of VOCs.^(28–30) Currently artificial olfaction has numerous industry applications, such as indoor air-quality monitoring⁽³¹⁾, medical care^(5,32), customs security⁽³³⁾, food quality control^(34,35), military applications^(5,36), and hazardous gas detection^(26, 37).

1.2. Mammalian olfaction and the basis of the 'electronic nose'

The 'electronic nose' device mimics the mammalian olfactory system which is able to detect and recognise a variety of different odorant molecules (Figure 1). Mammals have approximately 1,000 genes encoding different odorant receptor proteins situated in their olfactory organ.^(26, 38) Each receptor protein is capable of recognising multiple odorants.^(39, 40) In addition, a single odorant may be recognised by multiple protein receptors.^(39, 40) The odorant is then recognised in the mammalian brain by the combined pattern of the activated receptors.⁽³⁹⁾

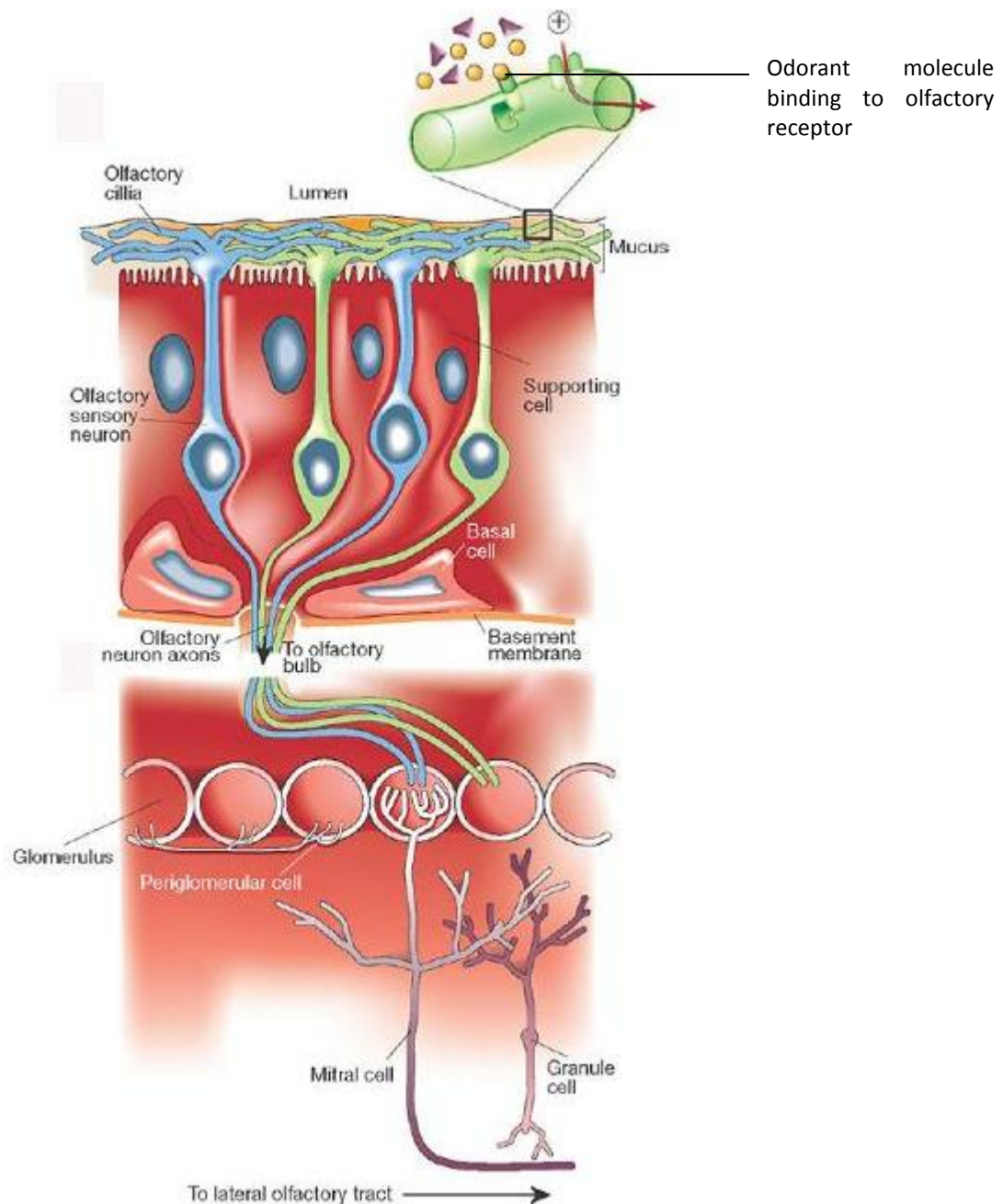


Figure 1. Organisation of the olfactory system.⁽⁴¹⁾ An olfactory receptor is a G-protein coupled receptor which detects odorants in order to generate the sense of smell. These receptors (olfactory sensory neurons) are situated in the dorsal-posterior nasal cavity epithelium. To distinguish different odorants, each olfactory sensory neuron expresses only one type of odorant receptor. Activation of an odorant receptor evokes action potentials in the olfactory sensory neurons, which relay information to the olfactory bulb in the brain.

The mammalian olfactory system does not detect an odour by using just one sensor, but rather an array of multiple receptor cells, with each combination sensing a different odour, that represents an odour signature.⁽²⁶⁾ Thus mammals use numerous permutations and combinations to distinguish many different odours. A similar system has been adopted for the electronic nose which uses “non-selected sensors to form the sensor array” which is used to detect odour, generate and identify the odour “fingerprint”.⁽²⁶⁾ However, the number of sensors for most electronic systems is limited; “sensors typically numbering between several and several tens are often chosen depending on the application”.⁽²⁶⁾

In brief, once an odour molecule has bound to an odour receptor/s, the receptor undergoes conformational changes, and binds and activates the G-protein on the olfactory receptor neuron (Figure 2).⁽⁴²⁾ The G-protein activates adenylylase cyclase which converts adenosine triphosphate to cyclic adenosine monophosphate (cAMP).⁽⁴²⁾ The cAMP opens calcium and sodium channels, allowing an influx of these ions and resulting in depolarisation of the sensory neuron.⁽⁴²⁾ An action potential is generated resulting in complex signals that the brain is able to interpret through a complex system as a particular odour.⁽⁴²⁾

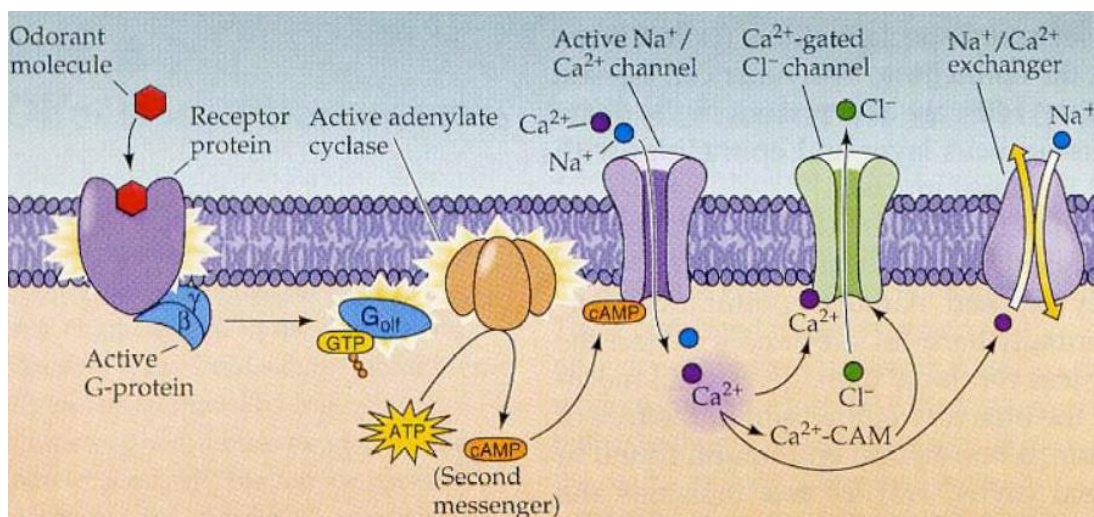


Figure 2. Schematic of signalling pathway.⁽⁴¹⁾

Key: Ca^{2+} calcium ion, Cl^- chloride ion, Na^+ sodium ion, ATP adenosine triphosphate, cAMP cyclic adenosine monophosphate, GTP-guanosine 5'-triphosphate, CAM calmodulin.

The sensor array in a chemiresistive sensor works similar to the olfactory nerves in the human olfactory system (Figure 3).⁽²⁸⁾ The 'electronic nose' system typically consists of three functional components: a sample handler (vacuum/gas pump), a multisensor array, and a signal processing unit such as an artificial neural network, software with digital pattern-recognition algorithms, and reference-library databases.^(29, 30, 43) In brief, once an odour molecule binds to a receptor/s on the sensory array of a chemiresistive sensor, the sensor films swell/shrink causing a change in resistance.⁽²⁶⁾ This change in resistance is used by software within a computer to "recognise" the odour using pattern recognition algorithms.⁽²⁶⁾ The devices are thus engineered to "mimic the mammalian olfactory system within an instrument designed to obtain repeatable measurements", allowing identification and classifications of aroma mixtures.⁽²⁶⁾

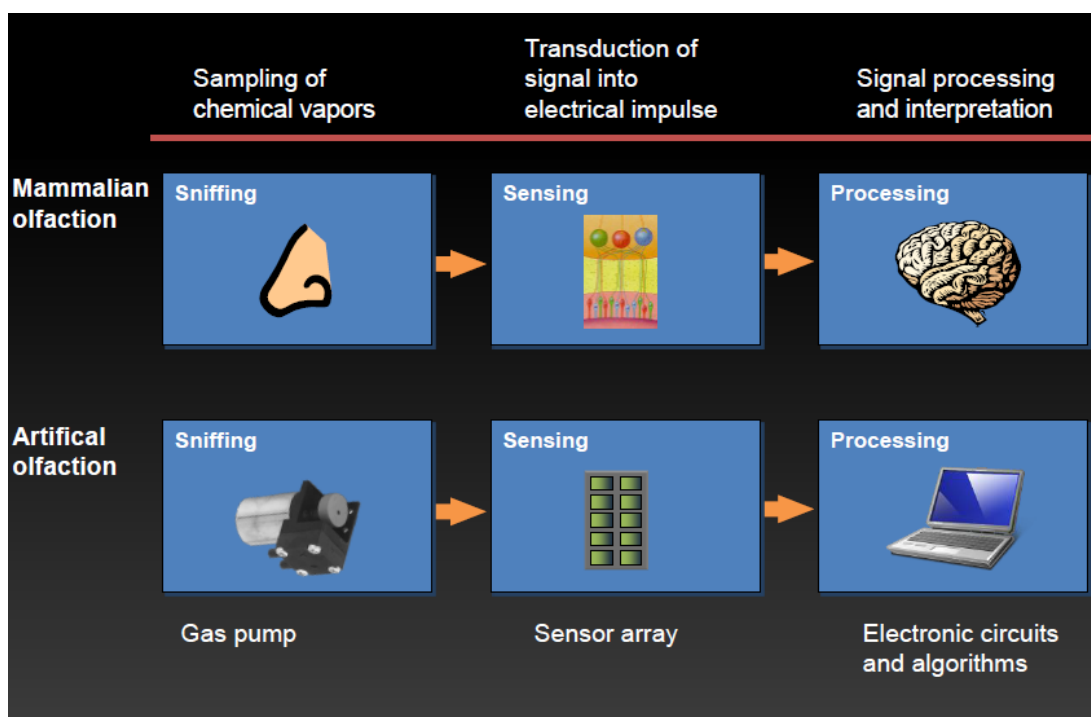


Figure 3. Artificial olfaction based on mammalian olfactory system. (Image courtesy of Dr William Royea, NextDimension Technology).

The sensor array consists of different sensors which react to a wide range of chemical classes and discriminate varied mixtures of possible analytes.^(28, 44) The sensor is the key element of which there are five categories: conductivity sensors, piezoelectric sensors, MOSFETs (metal-oxide semi-conductor field-effect transistor), optical sensors, and spectrometry-based sensing methods.⁽⁴³⁾ There are two types of conductivity sensors: metal oxide and polymer, both of which exhibit a change in resistance when bound to VOCs.⁽⁴³⁾ However the number of sensors that can be fitted to an electronic system is limited.

In a typical electronic nose, an air sample is pulled by a vacuum pump through a tube (made of plastic or stainless steel) into a small chamber housing the electronic sensor array.⁽⁴³⁾ Each sensor is then driven to a known

state by having clean, dry air or some other reference gas passed over its active elements.⁽⁴³⁾ The sample-handling unit then exposes the sensors to the odorant, producing a transient response as the VOCs interact with the surface and bulk of the sensor's active material.⁽⁴³⁾ A steady-state condition is reached in a few seconds to a few minutes, depending on the sensor type.⁽⁴³⁾ During this interval, the sensor's response is recorded and delivered to the signal-processing unit.⁽⁴³⁾ An optional washing step may be added, where a gas such as an alcohol vapour is applied to the array for a few seconds to a minute, so as to remove the odorant mixture from the surface of the sensor's active material.⁽⁴³⁾ The final step is application of the reference gas to the array, to prepare it for a new measurement cycle.⁽⁴³⁾ The period during which the odorant is applied is called the "response time" of the sensor array.⁽⁴³⁾ The period during which the washing and reference gases are applied is referred to as the "recovery time".⁽⁴³⁾

The outputs from the individual sensors are collectively assembled and integrated to produce a distinct digital pattern (an electronic fingerprint).^(28, 44) The identity of a simple or complex mixture represented by a unique pattern may be determined without having to separate the mixture into its individual components.^(28, 45) A database of digital aroma signature patterns for known samples is constructed prior to analysis of unknown samples.^(28, 45) The artificial neural network is configured through a learning process using pattern recognition algorithms.⁽²⁸⁾ This process continues until "a previously selected level of discrimination is met".⁽²⁸⁾ The results are "validated and assembled into the reference library to which unknown samples can be compared."⁽²⁸⁾ The "identification of unknowns is based on the distribution of elements that the analyte pattern has in common with patterns present in databases of the reference library."⁽²⁸⁾

1.3 The test device

Our collaborators at NextDimension Technology, Inc. (California, USA) have developed a device that involves the use of arrays of insulating, chemically sensitive films containing a dispersion of electrically conductive particles. Using a previously described evaporative deposition process, a series of 16 unique chemiresistive sensor elements were deposited onto a custom FR-4 based substrate.⁽⁴⁶⁾ The materials that comprised the sensor array were selected based on the measured ratio of their sensitivity towards published biomarkers of TB, including derivatives of benzene, naphthalene, and alkanes, relative to that of water vapour.⁽⁴⁷⁾

When exposed to chemical vapours, the individual sensor films swell, decreasing the number of connected pathways between conducting particles, causing a rapid and reversible resistance change (Figure 4).

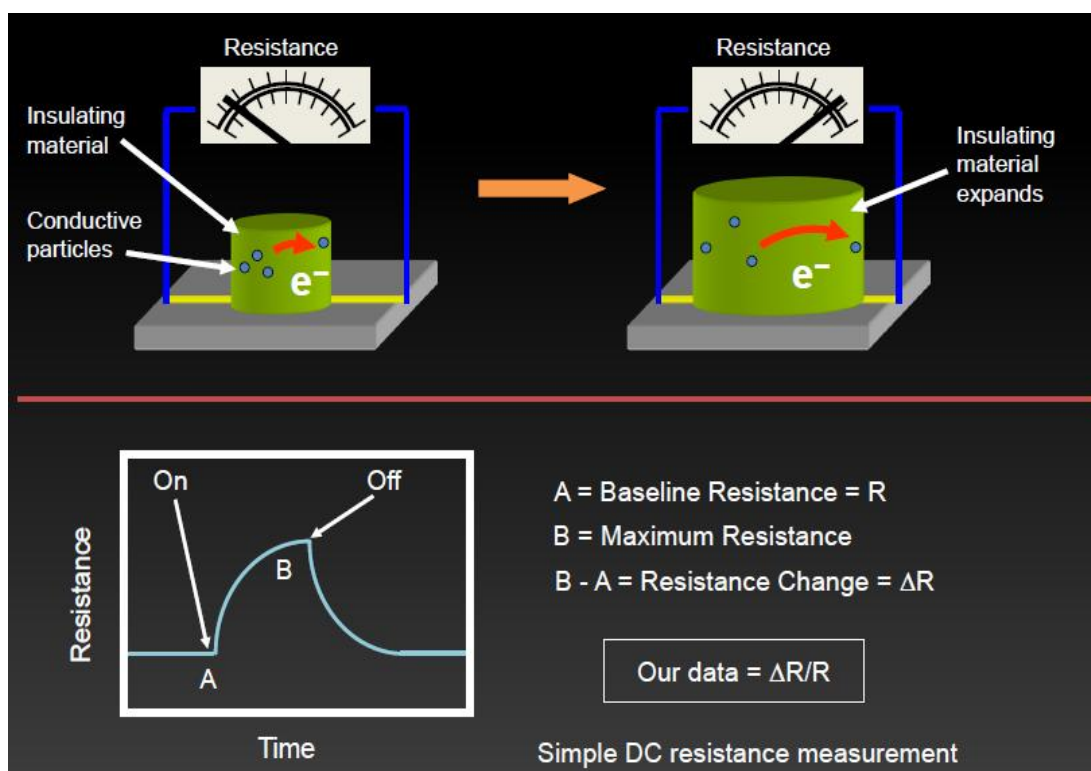


Figure 4. Image depicting how change in resistance is detected by the sensor. (Image courtesy of Dr William Royea, NextDimension Technology)

The combination of resistance changes over 16 sensors produces distinct fingerprints of analytes or mixtures of analytes, allowing for recognition of the analytes (in this case the volatile biomarkers associated with TB) using pattern recognition algorithms (Figure 5).

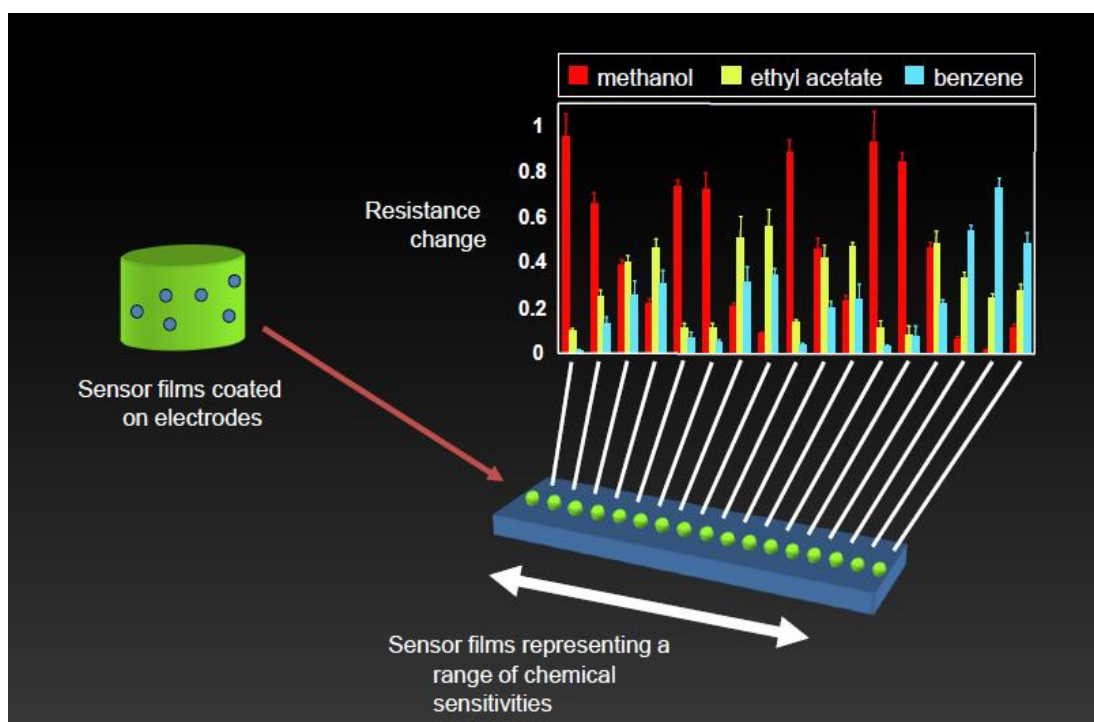


Figure 5. Differentiation between analytes using sensor arrays. An array of broadly cross-reactive sensors in which each individual sensor responds to a variety of analytes (bottom-right); a pattern of differential responses across the array produces a unique pattern for each analyte or analyte mixture (top right). (Image courtesy of Dr William Royea, NextDimension Technology).

The chosen sensors (Figure 6) are particularly sensitive to low vapour pressure analytes which absorb strongly into the chemiresistive films, yielding detection limits in the low parts per billion (ppb) and parts per trillion (ppt) range. In addition, the sensors are inherently compatible with simple, inexpensive digital electronics, and they operate at ambient temperature and pressure.



Figure 6. Image of the sixteen sensors of the test device. (Image courtesy of Dr Will Royea, NextDimension Technology)

The films utilised are based on an extensive body of work on semi-specific chemiresistors, which have yielded sensor arrays with unmatched sensitivity towards specific functional groups such as organic acids, thiols, and amines.⁽⁴⁸⁻⁵⁹⁾ For many analytes, such sensors can offer up to a tenfold increase in sensitivity relative to the early generation polymer composites (*communications with Prof NS Lewis, Caltech*). In addition, it has been shown that by deliberately placing sensors in spatially non-equivalent positions relative to the flow path of sampled analytes, additional kinetic information can be obtained that allows for the resolution of complex mixtures.^(56, 60)

1.4 Breath sensors as a TB diagnostic test

Studies^(3, 47, 61) using gas chromatography/mass spectrometry (GC/MS) have shown that unique combinations of VOCs, mostly branched and cyclic alkanes, and certain benzene derivatives, appear to have characteristic concentration patterns in cases of active PTB. A unique combination of these VOCs have also been detected in the headspace gas above *M. tuberculosis* cultures.⁽⁴⁷⁾ The compounds of interest are generally found at ppb to ppt levels in exhaled healthy human breath, but can be seen in “distinctive mixture compositions at 1-100 ppb (picomolar-nanomolar concentrations) in the breath of diseased patients.”⁽⁴⁷⁾ No one VOC serves to provide a robust diagnosis, but the pattern of approximately 12 principal components from 134 breath VOCs in combination can provide reasonable performance as a diagnostic screen for TB.⁽⁴⁷⁾ Phillips *et al.*⁽⁴⁷⁾, who employed these 12 principal components in their study, reported a 100% sensitivity and specificity using pattern recognition analysis of breath VOCs for discriminating healthy controls from hospitalised patients with suspected TB and a 82.6% (19/23) sensitivity and 100% (18/18) specificity when discriminating participants with positive sputum TB cultures from those with negative sputum cultures.

It has also been shown that testing of breath using GC/MS can be used to distinguish TB-infected patients from healthy controls using discriminant analysis.⁽⁶²⁻⁶⁴⁾ In a multicentre study⁽⁶²⁾ conducted in the Phillipines, United Kingdom and India involving 130 participants with active TB and 121 controls, Phillips *et al.* reported a sensitivity and specificity of 71.2% and 72%, respectively. In this study, the control group consisted of participants older than 13 years who were undergoing screening for PTB without clinical evidence of active disease.⁽⁶²⁾ Kolk *et al.*⁽⁶³⁾, using a classification model with

seven compounds, reported a sensitivity of 72%, specificity of 86% and an accuracy of 79% compared with culture. Their test device was validated using breath samples from 21 culture-positive TB and 50 culture-negative, non-TB participants.⁽⁶³⁾ In this study, a non-TB participant was defined as a participant with a cough for longer than two weeks, negative microbiology results (smear and culture) and having not received antituberculosis treatment.⁽⁶³⁾ Van Beek *et al.*⁽⁶⁴⁾, using nitric oxide as a screening test, reported a sensitivity of 78% (95% CI [68, 86]) and specificity of 62% (95% CI [47, 75]) using healthy hospital workers as the control group. Fend *et al.*⁽⁶⁵⁾, using electronic nose technology, were able to discriminate *Mycobacterium bovis* infected badgers (*Meles meles*) from healthy controls as early as three weeks post infection.

Although the technology looks promising from the previously mentioned studies, a limitation of some of these studies is that the control groups were healthy individuals, who do not represent the target population (i.e. patients suspected of having active PTB). Ideally, a group consisting of participants with respiratory symptoms but who were culture-negative for TB, is required to assess the true utility of breath-based testing. Additionally, each of the sensors in the aforementioned studies was based on different technologies performed in different research settings, often detecting differing VOCs, thus making comparison of the technology difficult.

Many of these tests were initially carried out in special labs with highly trained technical staff and expensive equipment using GC/MS.^(47, 63) However, due to advances in sensor technology over the last 10 years, similar testing can be done rapidly, relatively inexpensively, and potentially be performed directly at the clinic.⁽⁶²⁾

We sought to investigate the use of such a sensor based on nanotechnology that was specifically developed by our collaborators for POC testing for diagnosis of active PTB. Furthermore, we wished to show proof-of-concept by testing the device in the field. For this reason, we conducted a prospective study at several primary healthcare clinics in the Drakenstein region of the Western Cape, South Africa. As opposed to other studies performed previously, the test was performed by a primary healthcare nurse/layperson clinical research worker and the data were captured in real-time.

2. AIMS

2.1 To assess the sensitivity and specificity of a breath-based screening device at POC for the rapid detection of PTB amongst participants with a high clinical suspicion of PTB in comparison to healthy controls.

2.2 To assess the sensitivity and specificity of the device amongst participants with a high clinical suspicion of PTB who are microbiologically confirmed as TB-positive compared to those who are microbiologically confirmed as TB-negative.

3. METHODOLOGY

3.1 Clinical sites

This prospective study was conducted between August 2010 and March 2011 at five primary healthcare outpatient clinics in the Klein Drakenstein region of the Western Cape, South Africa: Phola Park, JJ Du Pré Le Roux, Wellington, Mbekweni and Dalvale clinics. Healthy volunteers were recruited from the staff of the microbiology diagnostic laboratory at Groote Schuur Hospital, National Health Laboratory Services (NHLS), Cape Town.

3.2 Participants

3.2.1 Healthy volunteers (control group)

Inclusion criteria

The inclusion criteria for the healthy volunteers were:

- age over 18 years, and
- ability to give informed consent.

Exclusion criteria

- Any symptoms suggestive of a respiratory tract infection or active TB. These symptoms included: cough, night sweats, fever, fatigue, weight loss, haemoptysis, dyspnoea; or symptoms suggestive of an upper respiratory tract infection, such as sore throat, rhinitis, etc.

3.2.2 Participants suspected of having TB (TB suspect group)

Inclusion criteria

The inclusion criteria for the participants suspected of having TB were:

- ≥ 18 years of age;
- ability to give informed consent;
- cough (of any duration) and/or any one of the following symptoms suggestive of PTB: haemoptysis, dyspnoea and/or constitutional symptoms of TB such as night sweats, loss of weight, and fever; and
- the ability to provide two sputum specimens for microscopy and culture and /or Xpert®MTB/RIF assay (Cepheid, Sunnyvale, California, USA).

Exclusion criteria

- Too ill to perform an exhalational manoeuvre (any patient with respiratory distress or with a resting respiratory rate ≥ 30 breaths/minute).
- Currently on TB treatment for longer than 48 hours (i.e. taking any of the following antituberculosis drugs: isoniazid, rifampicin, ethambutol, pyrazinamide, amikacin, streptomycin, kanamycin, capreomycin, moxifloxacin, or ofloxacin).

Participants were either recruited when they attended the local primary health clinic or they were co-recruited from another study investigating the

Xpert®MTB/RIF assay entitled “The impact of rapid molecular diagnosis of tuberculosis on tuberculosis services and patient care - a cluster randomised trial” (UCT Human Research Ethics Committee REF 366/2009).

In contrast to similar studies⁽⁶⁶⁾ performed previously, smokers were not excluded. This was done in order to simulate real world testing conditions where a significant number of individuals smoke cigarettes [rate in South Africa of 459 cigarettes per person per year per capita consumption⁽⁶⁷⁾]. In addition, smoking is a known risk factor^(68, 69) for acquisition of TB disease, and we therefore wished to evaluate the performance of the sensor in this group of high-risk individuals.

All eligible participants were screened by a standardised questionnaire which recorded the following information: participant demographics, current symptoms suggestive of respiratory tract infection or active PTB, recent contact with a TB-infected individual, previous TB infection, human immunodeficiency virus (HIV) infection status, as well as factors that potentially could interfere with VOC levels. These included smoking history, use of alcohol and illicit drugs, other medication, and any co-morbid medical illnesses. The participants were asked to refrain from smoking and consuming any food or beverages for two hours prior to the test. They were remunerated fifty South African rands for the inconvenience of fasting.

Two spot sputum specimens were collected from each of the participants suspected of having TB. These were sent for smear microscopy, mycobacterial culture and/or Xpert®MTB/RIF testing.

The participants suspected of having TB were divided into three groups for analysis based on microbiological test results:

- (1) smear-positive, mycobacterial culture /Xpert®-positive;
- (2) smear-negative, mycobacterial culture/Xpert®-positive; and
- (3) smear-negative, mycobacterial culture/Xpert®-negative group.

The smear-negative, mycobacterial culture/Xpert®-negative group were contacted at one month for follow-up (via telephonic or home visit) to ensure resolution of their symptoms without commencing TB treatment. Any participants with persistent symptoms were referred back to the clinic for further testing and were excluded from the study as TB could not be excluded as the cause of their persistent symptoms.

3.3 Equipment and test procedure

3.3.1 Breath collection bag

The breath test consisted of four main components: a GaSampler breath capturing bag (QuinTron, Milwaukee, USA), a sensor for the detection of the VOCs (NextDimension Technology), an analyser (Agilent Technologies, California, USA), and a laptop (Lenovo, Johannesburg, South Africa) with NDT Breath analyser software (NextDimension Technology) for capturing the sensor readings.

Alveolar breath samples were collected using commercially available 750mL breath collection bags. The breath collection device consisted of a mouthpiece attached to a T-piece, reservoir bag, and breath capturing bag (Figure 7). The open end of the T-piece was attached to the breath collection bag via a one-way valve. The one-way valve prevented backflow of breath into the mouth. The mouthpiece and valve offered minimal resistance to expiration, so that participants could provide breath samples without much effort or discomfort.

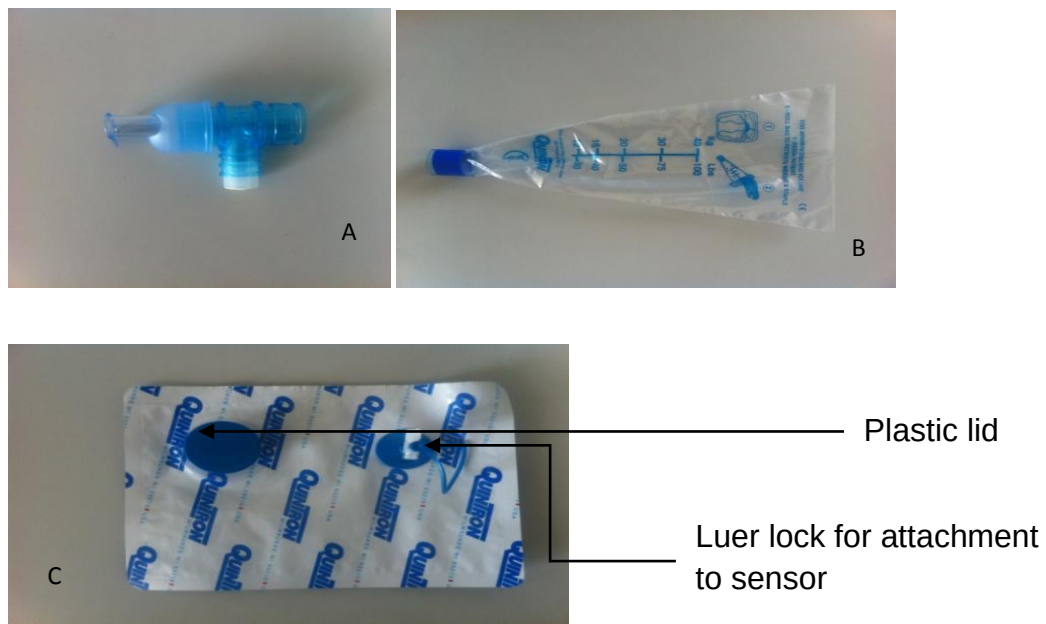


Figure 7. The components of the breath collection device.

Key: (A) mouthpiece; (B) reservoir bag; and (C) breath capture/collection bag.

Each participant was asked to breathe normally for two minutes, and then exhale into the mouthpiece (Figure 8). The exhaled breath was collected in a single exhalational manoeuvre. The reservoir bag filled first with 400 mL dead space air (which is the volume of inhaled air which does not take part in gas exchange because it remains in the conducting airways) and once the

pressure increased, alveolar air (which contains the gas in the pulmonary alveoli where oxygen/carbon-dioxide exchange with the pulmonary blood capillaries occurs) entered the breath collection bag via a one-way valve (next 750mL) [Figures 8 and 9]. Subjects unable to perform the required forced expiration manoeuvre were excluded from the study.

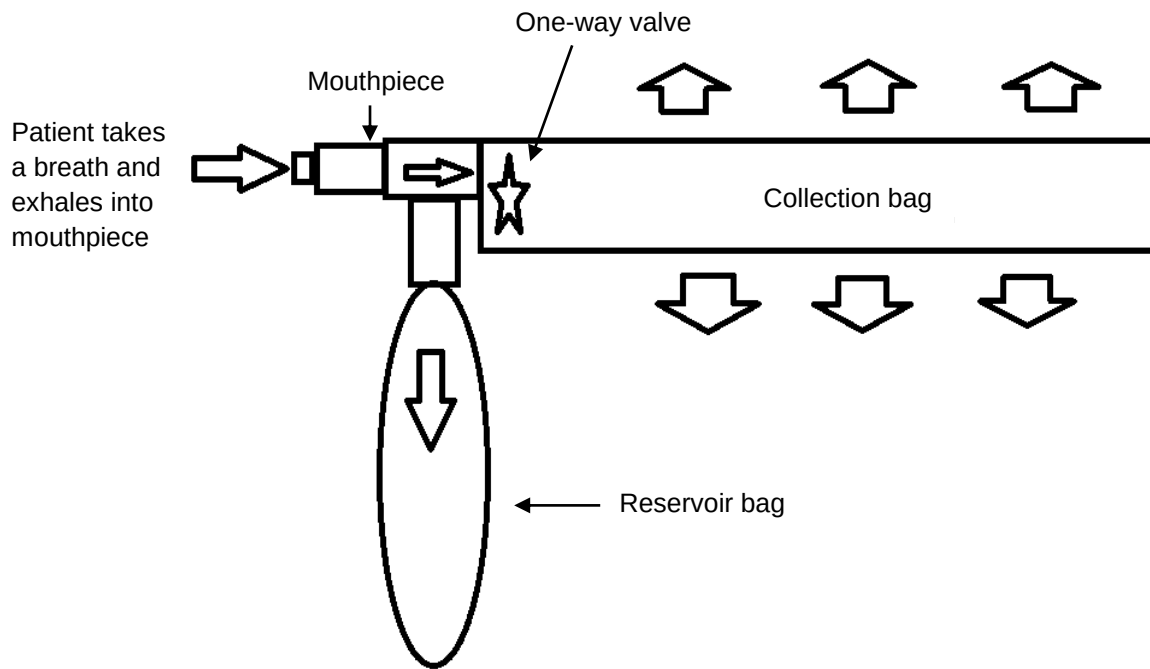


Figure 8. Breath collection bag. Arrows indicate flow of patient's breath, initially filling reservoir bag, then when pressure is exceeded, the one-way valve opens permitting the alveolar air of the patient's breath to collect in the collection bag.



Figure 9. Researcher exhaling into bag showing the full reservoir bag and breath collection bag slowly inflating.

3.3.2 VOC analysis

After breath collection, the mouthpiece and reservoir bag were detached, and the collection bag was connected to the front face of the sensor via a Luer lock, which was then opened. Following initialisation of the measurement software by the clinical operator, a sample of background room air was exposed to the sensors for one minute, followed by exposure for two minutes to the patient's breath sample, followed by a final exposure for an additional one minute of room air to clear the sensor array for the subsequent measurement. Air was flushed out via a male Luer lock on the exhaust port of the instrument. Once the test was completed, the bag was completely deflated using the bag preparation function of the sensor. The Luer lock was turned to the closed position and the bag was ready to be used for the next test. This process was repeated for each of the three breath samples provided by the test subject. The entire testing consumables (mouthpiece, reservoir bag, Luer locks, and breath collection bag) were discarded after each patient to prevent cross-transmission of any infectious agents.

The sensor array used in this study was integrated into a custom-designed, computer-controlled breath testing prototype detector that consisted of a 15 cm x 10 cm x 2 cm enclosure containing the sensor array, a custom sensor chamber fabricated from hard-anodised aluminium (180µL in volume), a micro diaphragm pump (operating at ~300 mL/min), a solenoid valve, teflon tubing connections, and a custom pump/valve control board. A diagram depicting the arrangement of the components in the detector is shown in Figure 10. All components in the flow path of the vapour were made of stainless steel, teflon, or hard-anodised aluminium.

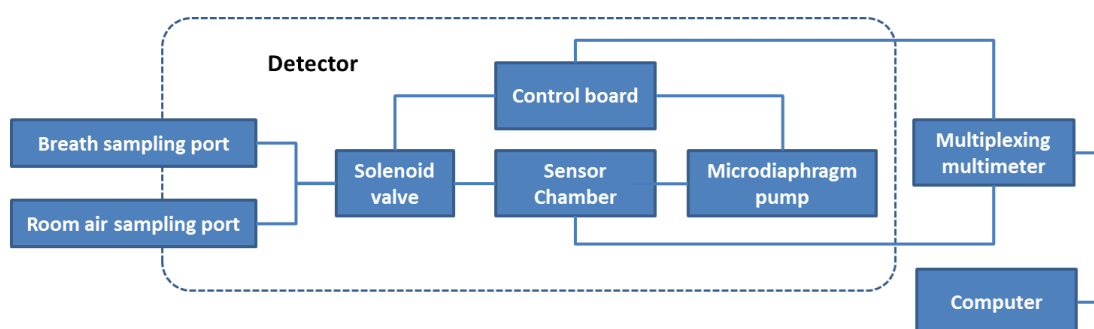


Figure 10. A schematic of the field-based breath sampling and detection system. (Courtesy of Dr Will Royea, NextDimension Technology)

The sensor was interfaced to an Agilent 34972A digital multimeter (Keysight Technologies, California, USA) equipped with an Agilent 34902A 16-channel multiplexer used to collect resistance data from the sensor array, and a 34907A multifunction module, used to control switching of the solenoid valve and pump. The multimeter was connected to a computer running custom LabVIEW software version 8.2.1 (National Instruments, Texas, USA) with a graphical user interface for use by the clinical operator. The front of the aluminium enclosure included three ports with Luer fittings: a breath

measurement port, a room air sampling port, and an exhaust port (Figure 11).

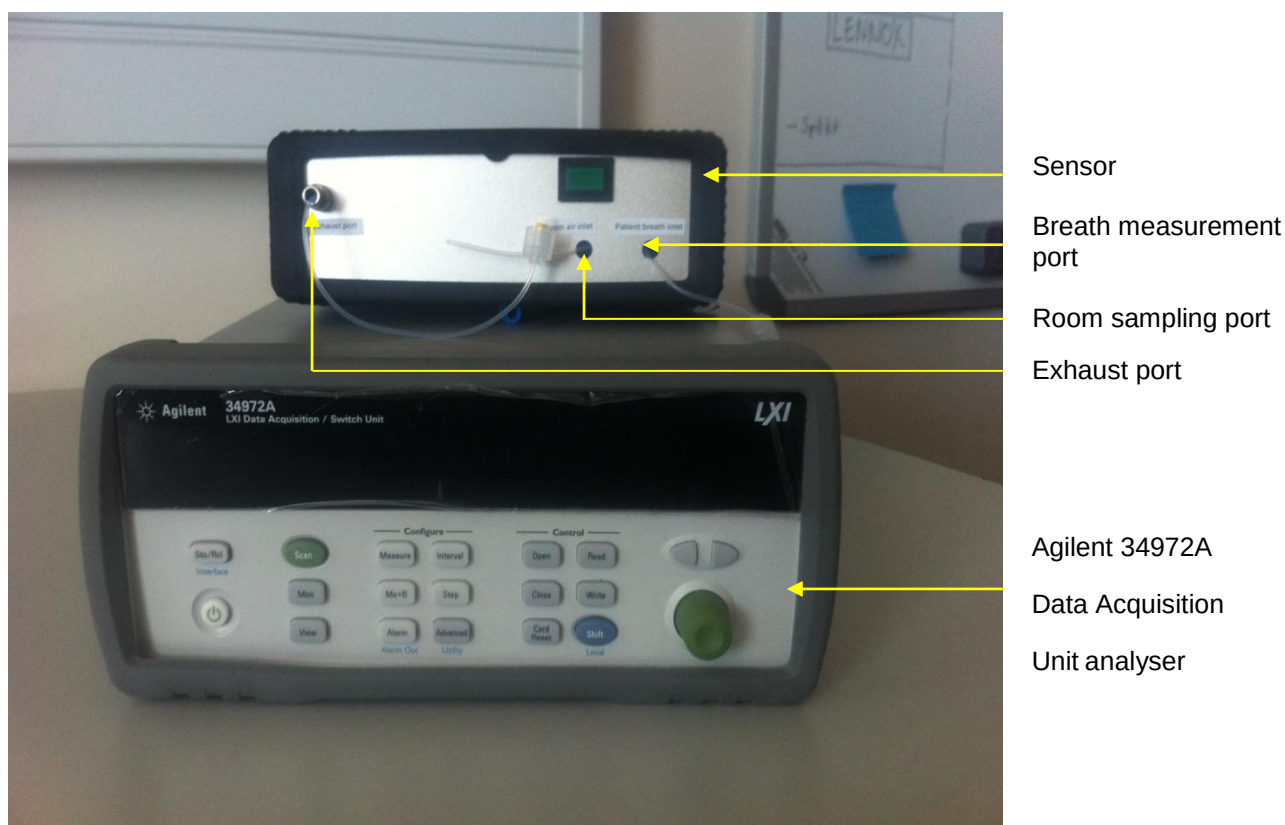
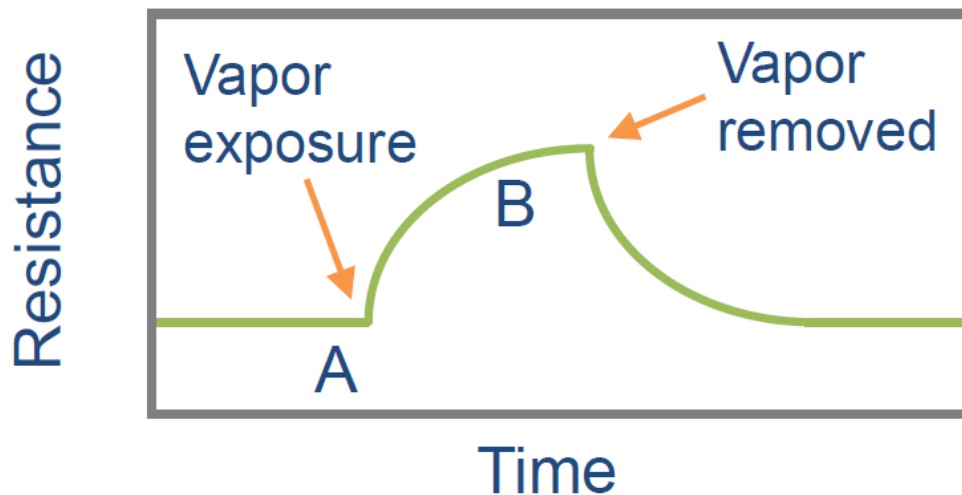


Figure 11. Sensor device showing sensor as well as Agilent data acquisition unit.

The sensor response for each sensor element is determined as the change in resistance following exposure to the analyte in the participant's breath. When the vapour (patient's breath) is removed, the resistance returns to its baseline resistance (Figure 12). Pre-exposure refers to the period (approximately two minutes) during which the sensor flushes environmental air across the sensor. Exposure refers to the period (approximately two minutes) during which the participant's breath sample is pumped across the sensors. During the remaining minute, the device empties the breath

collection bag and clears the sensors for the next measurement. Environmental air was used to flush the system between patient breaths as well as between patients.



A = Baseline resistance

B = Maximum resistance

$B - A = \text{Resistance change} = \Delta R$

Figure 12. Sensor response to exposure to a participant's breath and how change in resistance is determined. (Courtesy of Dr Will Royea, NextDimension Technology).

Although participants were asked to fast and refrain from smoking for two hours prior to the test, no particular restrictions regarding food, smoking, drug use, etc. were imposed on the participants.

The tests were performed in triplicate approximately five minutes apart and all three breath samples were collected during the same interaction with the investigator.

3.3.3 Software

Sensor readings were captured using the NDT Breath analyser programme using custom written code developed on Microsoft Visual studio (NextDimension Technology). The data from each participant was stored as a single file on a password protected computer.

3.4 Microbiology laboratory testing

All sputum samples were examined using either Ziehl-Neelsen/auramine staining and scored as per World Health Organisation guidelines at the NHLS Paarl Hospital laboratory. Auramine staining of concentrated sputum sediment and Mycobacterial Growth Indicator Tube (MGIT) culture (BACTEC MGIT 960, Becton Dickinson and Company, New Jersey, USA) was performed at the NHLS Greenpoint TB laboratory in Cape Town. The identification and susceptibility of cultured isolates were confirmed using the Hain MTBDR*plus* line probe assay (Hain Lifescience, GmbH, Nehren, Germany). The Xpert®MTB/RIF assay (Cepheid, Sunnyvale, USA) was performed at the NHLS Paarl laboratory. Positive microbiology results were relayed to the local clinic staff to ensure appropriate management of the affected participant. With the exception of two smear-positive patients who

were categorised as TB-positive due to reasons described later in the chapter, for the purposes of this study, either a positive TB culture or a positive Xpert® categorised a participant as TB-positive.

3.5 Statistical analysis

For each sensor channel, the resistance measurements were first corrected for baseline drift using the slope of the data obtained from the pre-exposure background using customised software described later in this chapter. After baseline correction, the relative differential resistance change ($\Delta R/R$) for each channel was calculated by taking the difference between the last data point during exposure (R_{MAX}) and the last data point during pre-exposure ($R_{BASELINE}$), then dividing by $R_{BASELINE}$. The $\Delta R/R$ values averaged over three breath sample measurements, $(\Delta R/R)_{AVG}$, were then sum-normalised by dividing each $(\Delta R/R)_{AVG}$ value from each sensor channel by the sum of all $(\Delta R/R)_{AVG}$ values from all channels.

Using the sum-normalised $(\Delta R/R)_{AVG}$ values, a linear discriminant analysis (LDA) approach was used to develop and test a predictive TB model using independent training and testing sets. Subjects from each group were randomly assigned (using a random number generator on Microsoft Excel) into equal size groups for either training or evaluative testing. Subjects with missing culture or breath test data were excluded from the analysis. Following development of a binary classification model using the training data, the model was applied to the test group, and classification statistics were determined.

Principal component analysis as well as LDA was performed using custom written software developed on Microsoft Visual studio by NextDimension Technology. The Kruskal-Wallis ANOVA test was used to determine statistical significance between the three participant groups for age and CD₄ T-cell enumeration. Similarly the Fisher's exact test was used for gender, haemoptysis, current use of illicit drugs, and current use of antiretrovirals (ART); whilst the Chi-squared test was used for the remaining variables in Table 1. These statistical tests were performed using IBM SPSS Statistics for Windows version 22.0 (IBM Corporation, Armonk, New York, USA).

3.6 Ethics approval

The study received ethics approval from the University of Cape Town Human Research Ethics Committee (HREC 378/2009). Approval from the Western Cape Provincial Department of Health was also obtained. All participants provided written informed consent prior to enrolment.

4. RESULTS

4.1 Healthy volunteers

Fifty healthy volunteers were recruited for the control arm.

4.2 Clinical characteristics and microbiological test results of participants suspected of having TB

The distribution of the enrolled participants is depicted in Figure 13. Twenty-five participants were excluded either due to technical problems encountered during testing of the participants, inability to provide two sputum specimens or remaining symptomatic at one month follow-up despite negative TB cultures or Xpert® testing.

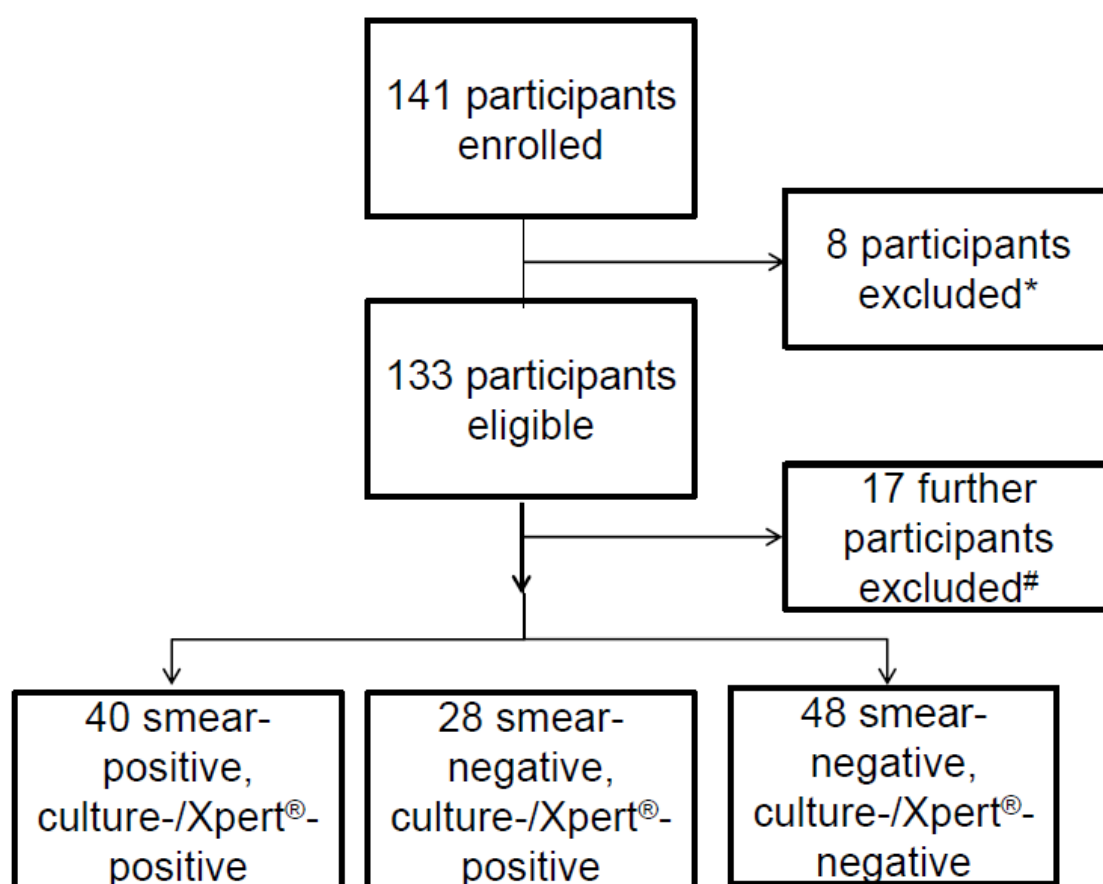


Figure 13. Distribution of the enrolled participants as per microbiological test results.

Key: * 8 participants excluded: 2 participants had errors with consent forms; 6 participants were on TB treatment >48 hours at time of testing ; # 17 participants excluded at end of study: 8 participants had incomplete microbiological testing performed; 1 participant died prior to 2 month follow-up; 5 participants cultured non-tuberculous mycobacteria, 1 participant did not have 3 tests performed due to technical error with the test device, and 2 participants remained symptomatic at follow-up.

Note: 90/116 participants had an Xpert® test performed; 114/116 participants had TB culture performed.

The mean age of the participants was 35.7 [IQR 18-71] years. Females made up 58.6% of participants with a statistically significant difference between the three groups ($p<0.002$). Symptoms at time of testing are shown in Table 1. Presence of night sweats and weight loss were significantly different across the three groups ($p<0.001$). The differences between the three groups with

regards to smoking, consumption of alcohol and use of illicit drugs were not statistically significant.

Table 1. Clinical characteristics of different participant groups.

	S+C+ participants, n (%)	S-C+ participants, n (%)	S-C- participants, n (%)	p-value	Significant/Non-significant	Statistical Test
Total (n)	40 (100.0)	28 (100.0)	48 (100.0)			
Demographics						
Mean Age [SD]	35 [13.2]	38.4 [10.7]	34.9 [10.1]	0.218	Non-significant	Kruskal-Wallis test
Male Gender	24 (60.0)	13 (46.4)	11 (22.9)	0.002	Significant	Fisher's exact test
Symptoms of TB						
Cough	40 (100.0)	28 (100.0)	48 (100.0)	Not done	Not applicable*	Chi-square test
Night sweats	34 (85.0)	24 (85.7)	22 (45.8)	<0.001	Significant	Chi-square test
Weight loss	33 (82.5)	16 (57.1)	13 (27.1)	<0.001	Significant	Chi-square test
Haemoptysis	6 (15.0)	6 (21.4)	3 (6.3)	0.133	Non-significant	Fisher's exact Test
Previous TB						
Recent TB contact	13 (32.5)	11 (39.3)	14 (29.2)	0.662	Non-significant	Chi-square test
Current smokers	9 (22.5)	16 (57.1)	25 (52.1)	0.005	Significant	Chi-square test
Currently drink alcohol	12 (30.0)	12 (42.9)	12 (25.0)	0.264	Non-significant	Chi-square test
Current use of illicit drugs	10 (25.0)	11 (39.3)	18 (37.5)	0.357	Non-significant	Chi-square test
Current use of HIV-infected	6 (15.0)	1 (3.6)	3 (6.3)	0.272	Non-significant	Fisher's exact test
Mean CD4 T-cell count [SD]	15 (37.5)	17 (60.7)	13 (27.1)	0.015	Significant	Chi-square test
Currently on antiretroviral drugs	183 [166], 13 (86.7) 4 (10.0)	200 [133], 17 (100.0) 6 (21.4)	263 [128], 13 (100) 2 (4.2)	0.062 0.069	Non-significant Non-significant	Kruskal-Wallis test Fisher's exact test

* Cannot compute p-value as 100% of participants in all three groups were positive for cough

Key: S+C+ smear-positive, culture-/Xpert®-positive
S-C+ smear-negative, culture-/Xpert®-positive
S-C- smear-negative, culture-/Xpert®-negative
SD standard deviation

Thirty one per cent of the participants (36/116) were current smokers, 33.6% (39/116) reported regular use of alcohol, and 8.6% (10/116) reported use of illicit drugs (crystal methamphetamine, cannabis and methaqualone).

Forty-five of 116 (38.8%) of participants were HIV-infected with only 7.8% (9/116) unaware of their HIV status. CD4 T-cell counts were available for 43 HIV-infected participants. The average CD4 T-cell count was 214 [IQR 15-672] and 12 (26.7%) of the HIV-infected participants were on ART. The median CD4 T-cell count was not statistically different across the three groups ($p=0.062$).

Of the smear-positive participants, 18 (45%) participants were 3+ acid-fast bacilli (AFB) smear-positive on either of the two smear microscopies performed. Although TB culture nor Xpert® testing was not performed on two smear-positive participants due to an omission by study staff, they were both included in the study as both participants had two positive smears, clinical features suggestive of TB and resolution of their symptoms on antituberculosis therapy. The average time to detection of a positive mycobacterial culture for the smear-positive group was 10 days. Thirty-three of the 40 (82.5%) smear-positive participants (of those co-recruited from the Xpert® MTB/RIF study) had an Xpert® MTB/RIF test also performed, of which all were positive for *M. tuberculosis* complex (Figure 14). Two participants were confirmed to have multidrug-resistant (MDR) TB by line probe assay performed on cultured isolates.

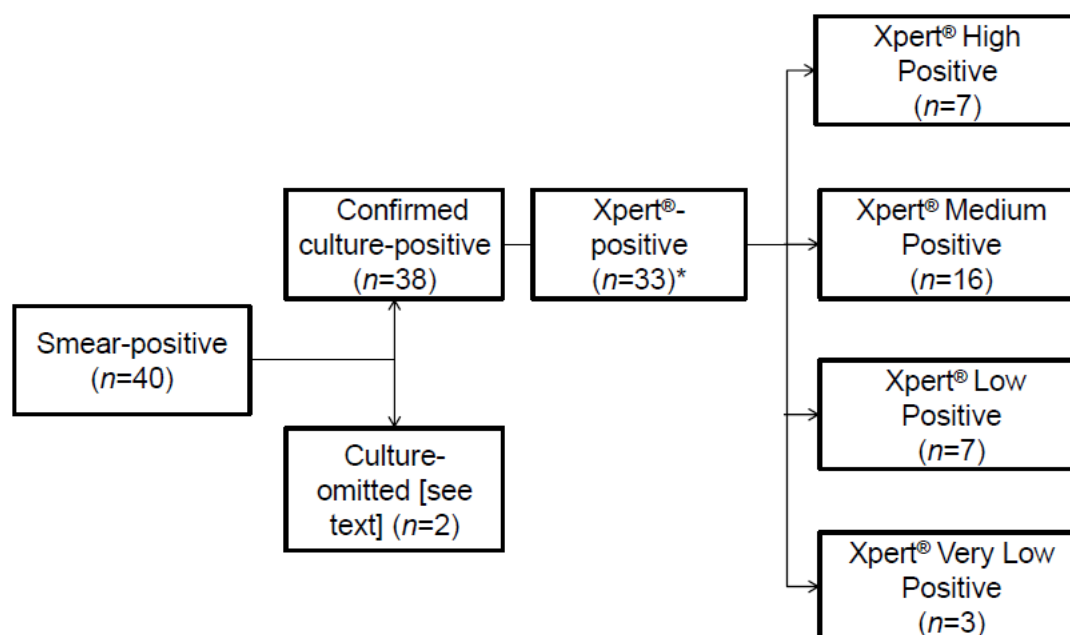


Figure 14. Microbiological results of the smear-positive, culture/Xpert®-positive group.

Key: * 7/40 smear-positive participants did not have Xpert® testing performed.

Of the sputum smear-negative, culture/Xpert®-positive group, 27 of the 28 (96.4%) participants had a positive TB culture (average positive within 16.9 days [IQR 8-29]). Twenty-three (82.1%) of the 28 participants had Xpert® MTB/RIF testing performed (Figure 15).

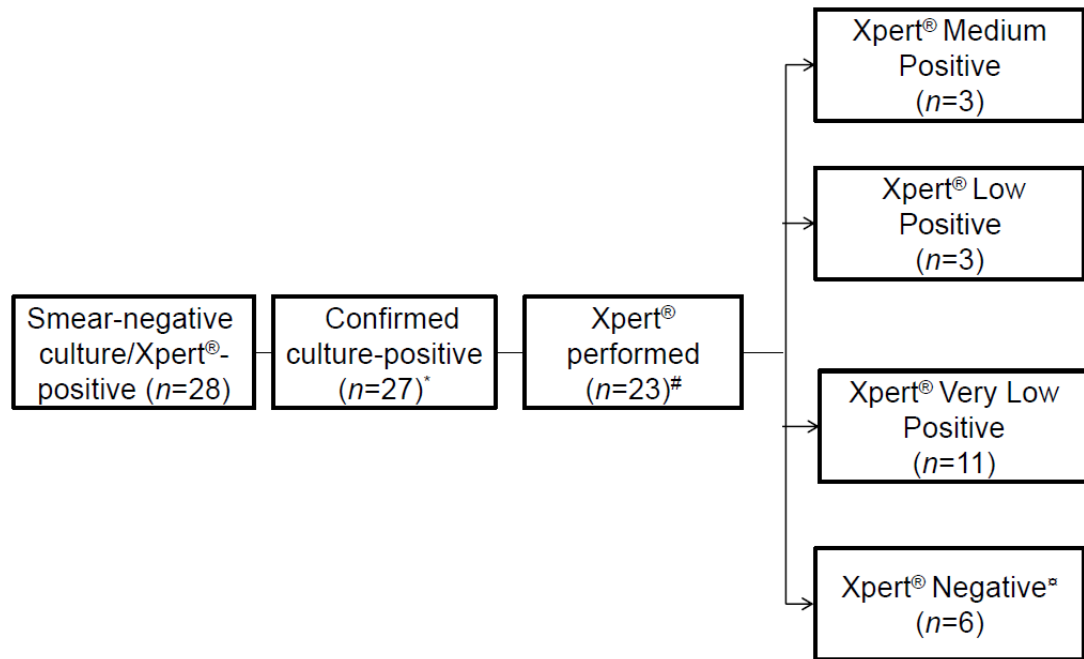


Figure 15. Microbiological results of the smear-negative, culture/ Xpert®-positive group.

Key: * One participant had a positive Xpert® only (insufficient sputum for culture); # Five participants didn't receive Xpert® testing (insufficient sputum for Xpert®); □ Six participants were Xpert®-negative, but culture-positive.

4.3 Sensor response profiles

Figure 16 shows a typical sensor-element response to a breath sample from a TB-positive (culture/Xpert®-positive) test subject.

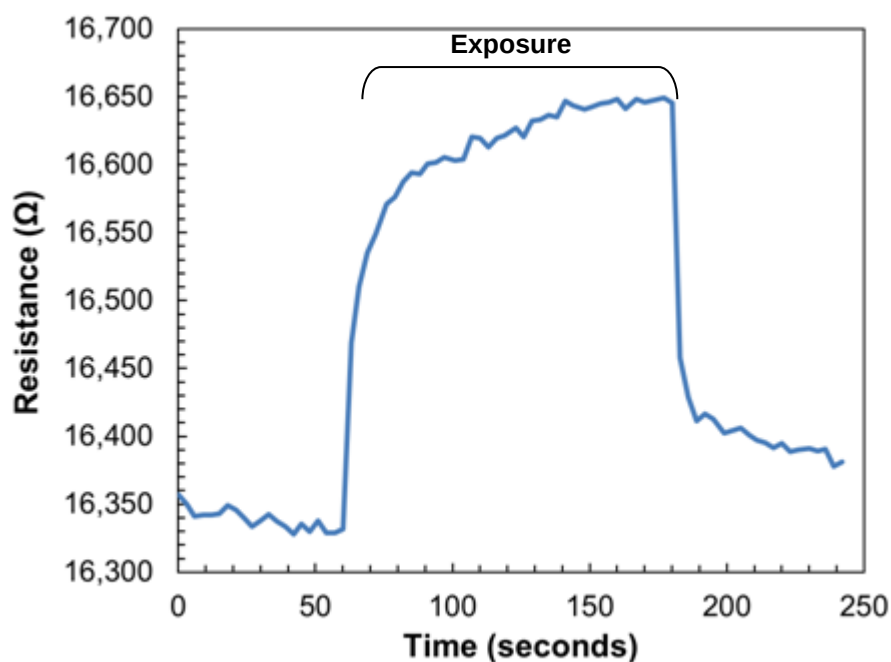


Figure 16. A representative sensor response from a single chemiresistive sensor element upon exposure to a breath sample from a TB-positive test subject. Exposure refers to the period (approximately two minutes) during which the participant's breath sample is pumped across the sensors.

Key: Ω ohm

In general, the sensors showed rapid and reversible behaviour with resistances reaching to within 10% of their steady-state response values within one minute of exposure to the breath sample and returning to within 10% of their baseline values within one minute following re-exposure to room air. Individual sensor-element responses were typically indistinguishable among healthy controls, culture/Xpert®-negative TB participants, and culture/Xpert®-positive TB participants (Figure 17). However, the combination of all 16 sensor profiles taken together, yielded on average statistically distinguishable sensor response profiles that could be used to differentiate the disease state from healthy controls. For many sensor elements, there were small statistical differences between the individual

sensors. However, there was insufficient information from any single given sensor to make a determination of disease state based on a single sensor reading, as it was the relative response of an individual sensor to other sensors that allowed for classification.

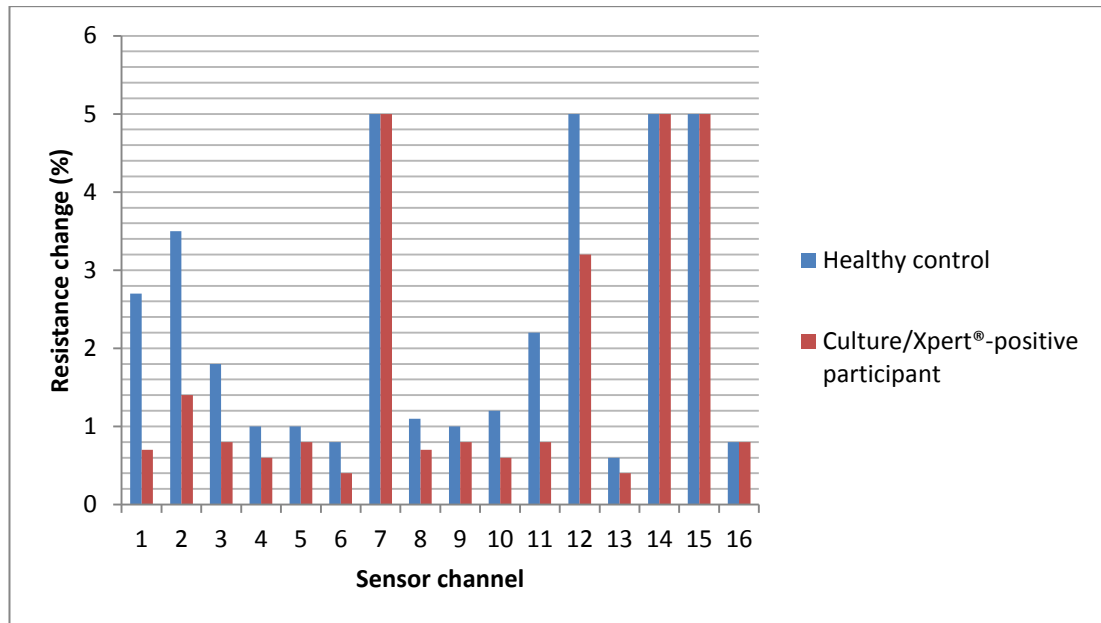


Figure 17. Average sensor responses across each channel for a healthy control and a TB-positive (culture/Xpert®-positive) participant. Sensor responses shown were obtained by averaging across each of the three breath measurements.

Randomisation into training/testing sets was performed five times and data obtained from these randomisations produced sensitivities that ranged from 97-100%, and specificities that ranged from 88-100%. A histogram of discriminatory function scores among all of the subjects in both the culture-positive and healthy control groups is depicted in Figure 18. Based on an LDA-derived optimal threshold established using the independent test set, active TB could be detected with 100% sensitivity and 92% specificity. Although the biomarkers targeted by the sensors included in this study are

the same as those targeted by Phillips *et al*⁽⁴⁷⁾, who reported a sensitivity of 100% and a specificity of 100% for results from their GC/MS study, it is impossible to prove whether we detected the same biomarkers due to the differing sensitivities of our device compared to conventional GC/MS. In addition, as our device is not able to separate VOCs individually, we were not able to confirm which VOCs represented ‘TB infected’ breath specimens as was shown in the GC/MS study.⁽⁴⁷⁾

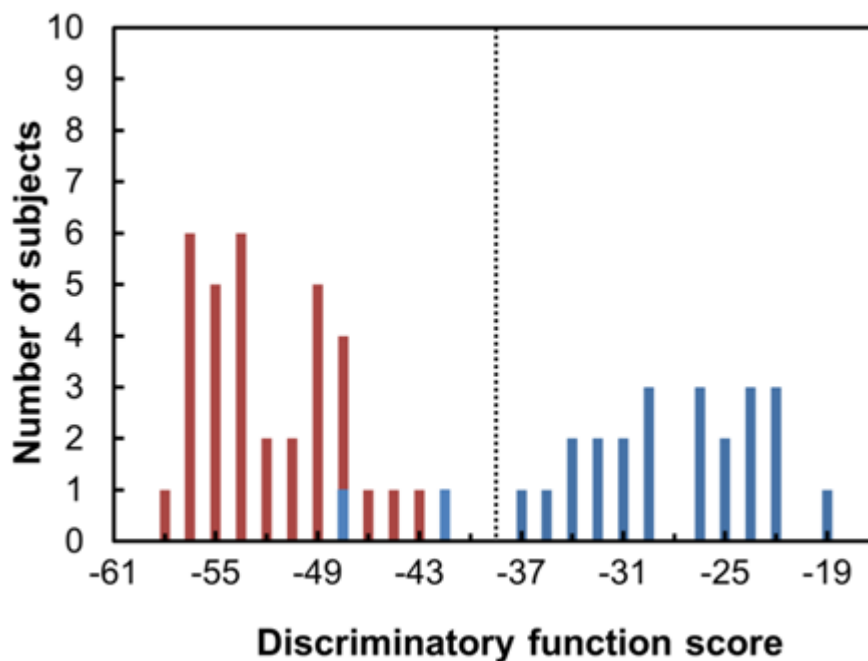


Figure 18. Histogram depicting linear discriminatory function scores amongst TB-positive participants (red) and healthy controls (blue) and the optimal discrimination line ascertained independently from the testing data (dashed-line).

Figure 19 shows the corresponding receiver operating characteristic (ROC) curve using the optimal threshold for the LDA with the independent, randomly selected training set.

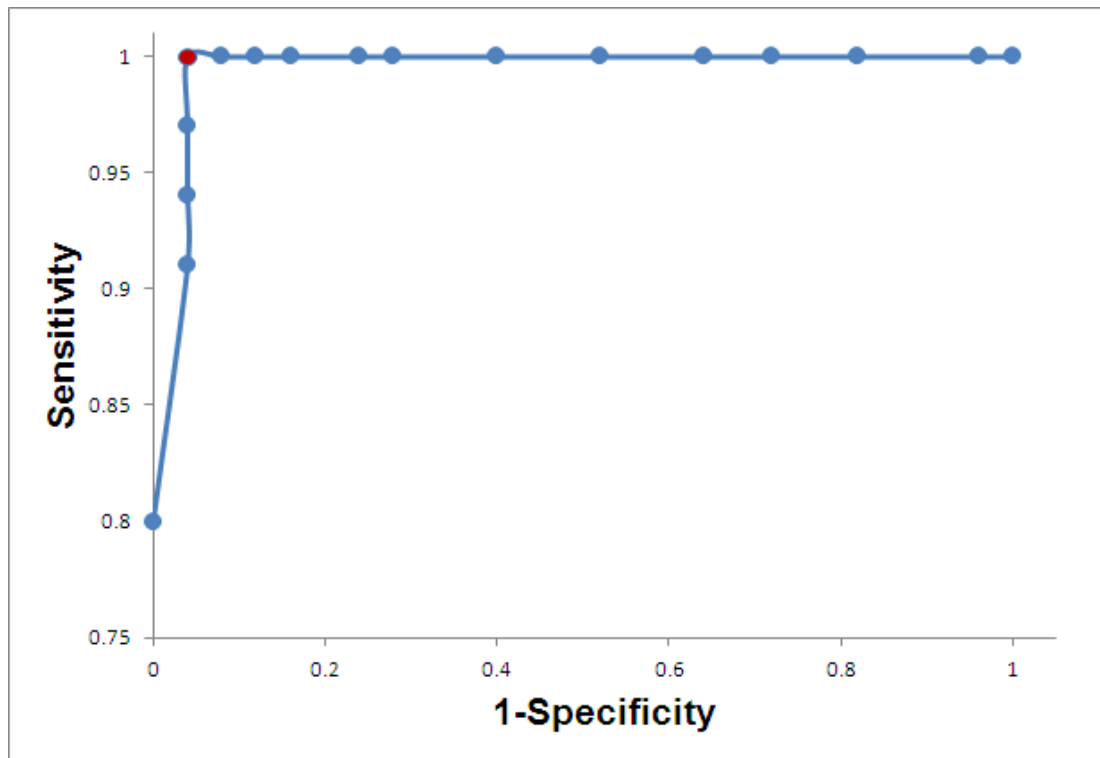


Figure 19. The receiver operating characteristic (ROC) behaviour for healthy controls relative to TB-positive test subjects. The sensitivity and specificity obtained using the optimal LDA threshold is indicated by the red marker.

The analogous histogram of discriminatory function scores for the culture/Xpert®-positive group vs. culture/Xpert®-negative group is depicted in Figure 20, and the corresponding ROC behaviour is shown in Figure 21.

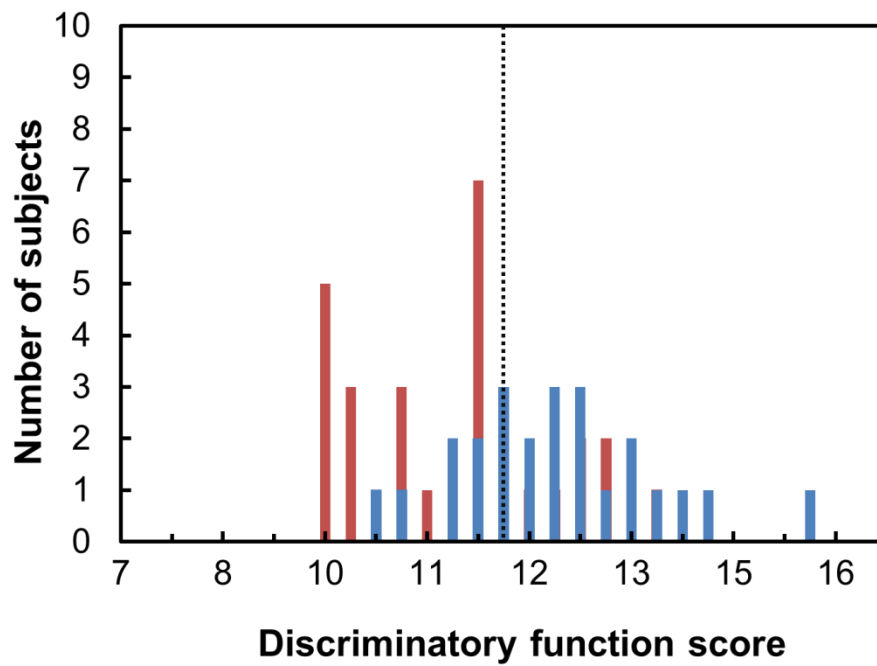


Figure 20. Histogram of LDA discriminatory function values (d-values) for culture/Xpert®-negative participants suspected of having TB (blue) and culture/Xpert®-positive participants (red) and the optimal discrimination line ascertained independently from the testing data (dashed-line).

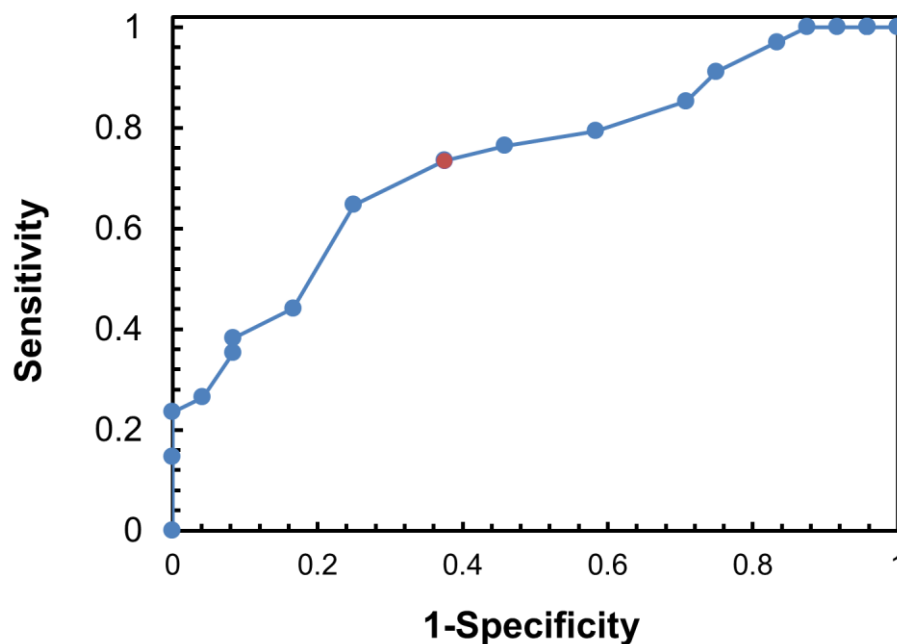


Figure 21. The receiver operating characteristic (ROC) behaviour for culture/Xpert®-negative participants relative to culture/Xpert®-positive test subjects. The sensitivity and specificity obtained using the optimal LDA threshold is indicated by the red marker.

In this case, a diagnostic sensitivity of 74% and a specificity of 63% were observed using the LDA-derived optimal threshold. A summary of additional classification statistics for various control and test groups obtained is listed in Table 2. The best results were obtained when comparing the confirmed TB-positive participant group to healthy controls. Although the sensitivity was decreased when compared with the culture/Xpert®-negative TB suspect group, the performance of the sensor was clearly better for the smear-positive, culture/Xpert®-positive group when compared to the smear-negative, culture/Xpert®-negative group.

Table 2. Summary of statistical results for different test and control groups using LDA thresholds ascertained independently from the testing data.

Control group	Test group	n _{CON}	n _{TEST}	True negatives	True positives	False negatives	False positives	Sensitivity (%)	Specificity (%)	LDA resolution factor
Healthy	Culture(+)	25	34	23	34	0	2	100%	92%	5.0
Healthy	Smear(+)/ Culture(+)	25	20	23	20	0	2	100%	92%	4.9
Healthy	Smear(-)/ Culture(+)	25	14	23	14	0	2	100%	92%	5.7
Culture(-)	Culture(+)	24	34	15	25	9	9	74%	63%	1.3
Culture(-)	Smear(+)/ Culture(+)	24	20	20	14	6	4	70%	83%	1.3
Culture(-)	Smear(-)/ Culture(+)	24	14	19	5	9	5	36%	79%	1.6

Key: n_{CON}- number in control group; n_{TEST}- number in test group; LDA- linear discriminant analysis; + (positive) ; - (negative); % (percentage).

Note culture (+) in the table above denotes a patient with either a positive TB culture or positive Xpert® result.

5. DISCUSSION

This pilot study was designed to establish proof of principle and obtain a preliminary estimate of the accuracy of this specific instrument and sensor. Our primary objective was to determine whether the sensor is sufficiently sensitive and specific to be used as a human diagnostic tool for active TB, and whether it could discriminate between clearly infected individuals and healthy controls. The results show that the sensor was able to accurately discriminate those with culture/Xpert®-confirmed TB from the healthy population, but performed relatively poorly in discriminating

culture/Xpert®-confirmed cases from participants with an alternative aetiology for their lower respiratory tract symptoms. Our results demonstrate, however, that this technique appears to be sufficiently promising to warrant further studies with an optimised device.

Our sensor demonstrated a high sensitivity (100%) and specificity (92%) for distinguishing patients with active TB from healthy controls. Bruins *et al.*⁽⁷⁰⁾ reported a sensitivity of 93.5% and a specificity of 85.3% in discriminating healthy controls from culture-confirmed TB patients in Bangladesh during validation testing of the DiagNose (Zutphen, Netherlands). Phillips *et al.*⁽⁶²⁾, in a multicentre-study conducted in the Philippines and India, reported a sensitivity of 71.2% and a specificity of 72% for a rapid POC device comparing participants suspected of having TB with controls. Their case definition was less rigorous than the previous study. Clinical disease was defined as presence of clinical suspicion of TB based on presence of signs and symptoms; or history of recent exposure to infection; or abnormal chest radiograph; or positive smear microscopy; or sputum culture-positive.⁽⁵²⁾ This may have accounted for the lower sensitivity and specificity reported as participants who may have been symptomatic with respiratory illnesses caused by pathogens other than TB could have been included in the clinical disease group. Our results are similar to those of Phillips *et al.*⁽⁴⁷⁾ who demonstrated better results in comparing sputum culture-positive patients with healthy controls than people at high risk of TB. The authors demonstrated that a set of VOCs accurately distinguished between normal controls and hospitalised patients, whilst a different set of VOCs was required to distinguish hospitalised patients whose sputum cultures were positive or negative for *M. tuberculosis*.⁽⁴⁷⁾ The ability of the sensor studied in our trial to discriminate truly infected from 'healthy controls' demonstrates that this technology has considerable promise as a rapid screening diagnostic.

A lower sensitivity (74%) and specificity (63%) was detected when the culture/Xpert®-negative participant group was compared to the culture/Xpert®-positive participants in our study. Bruins *et al.*⁽⁷⁰⁾ reported a sensitivity of 76.5% and specificity of 87.2% for their device when identifying TB patients within the entire test-population (best-case numbers). Kolk *et al.*⁽⁶³⁾ found a sensitivity of 72% and specificity of 86% in a South African cohort comparing culture-confirmed TB participants to culture-negative participants. Of note, Kolk *et al.*⁽⁶³⁾ used GC-MS in their study to differentiate between TB and non-TB breath samples. GC-MS is considered the reference standard for VOC discovery.⁽⁸⁾ The VOC technology utilised in our device is not as powerful as GC-MS in separating and identifying VOCs and this may explain the lower specificity in our study compared to the results of Kolk *et al.*⁽⁶³⁾

There are several possible explanations for the limitations of sensitivity and specificity reported. Firstly, an individual's breath contains hundreds of compounds with combinations that are usually distinctive of an individual.⁽⁷¹⁾ This accounts for the inherent difficulty of breath testing as these compounds may interfere with detection of VOCs generated by the bacterium. The unique microbiome of the host may additionally contribute to VOCs that are very similar to those detected by the sensor making it difficult for the sensor to discriminate between colonising and pathogenic bacteria.⁽⁸⁾

It is also possible that the lower specificity may be partly accounted for by the choice of the VOCs that were incorporated into our sensor. Although pathogenic bacteria are capable of producing a wide variety of VOCs, only a fraction of VOCs are produced exclusively by a bacterial species.⁽⁸⁾ Therefore, it is possible that patients with other bacterial respiratory tract infections

could produce VOCs similar to *M. tuberculosis*, and therefore account for the lack of specificity of our sensor. Little is known about the generation of VOCs from other potential pulmonary pathogens (apart from *Pseudomonas aeruginosa* and *Aspergillus fumigatus*) due to the paucity of research in this field.^(10, 15) In a recent systematic review, Bos *et al.*⁽⁸⁾ demonstrated overlapping VOC generation by bacteria when comparing VOCs generated by gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis*) against those generated by gram-negative organisms (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) [Figure 22].⁽⁸⁾ The authors found that only a small fraction of metabolites are produced exclusively by a single bacterial species.⁽⁸⁾ Additionally, the immune response produced by the host may interfere with the metabolic pathways of the bacterium, and therefore also influence VOC generation by the pathogen.⁽⁸⁾

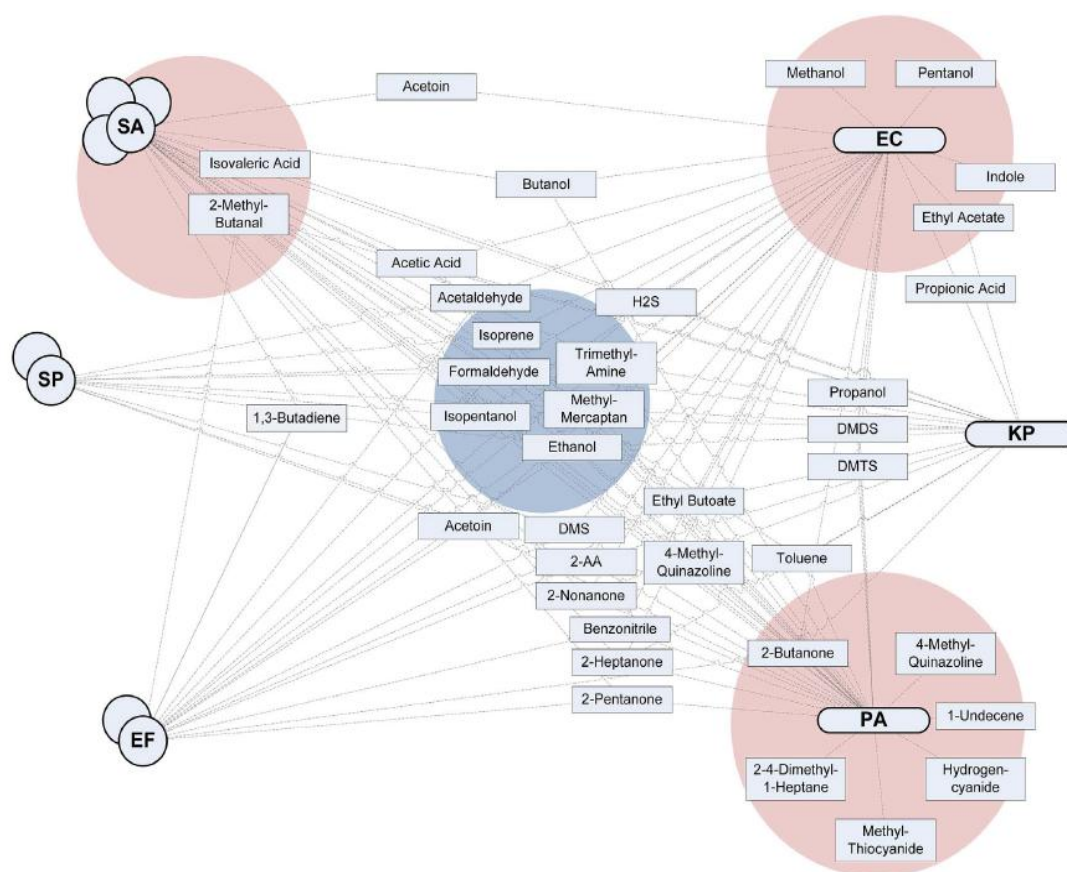


Figure 22. Interaction plot demonstrating overlapping metabolites produced by different bacteria.⁽⁸⁾ Gram-positive bacteria are demonstrated on the left with gram-negative bacteria on the right. The blue zone indicates metabolites that are (almost) always produced by all pathogens; the red zones indicate metabolites that are produced by only one or mainly one strain of bacteria.

Key: SA: *Staphylococcus aureus*, SP: *Streptococcus pneumoniae*, EF: *Enterococcus faecalis*, EC: *Escherichia coli*, KP: *Klebsiella pneumoniae*, PA: *Pseudomonas aeruginosa*

Thirdly, some of the VOCs that were detected may be host-response related VOCs, rather than pathogen-specific markers, and this could account for the lack of specificity of the sensor.^(8, 71) The inflammatory response alters host metabolism and this may affect VOC generation. It is therefore possible that patients with other infections (non-TB infections) or non-infectious

inflammatory conditions may produce the same/similar VOCs in their lungs as those associated with active TB.

Fourthly, the VOCs that were primarily targeted by our device were based on the signature detected by Phillips *et al.* when *M. tuberculosis* was grown *in vitro*.⁽⁴⁷⁾ It is possible that the VOCs produced in culture media may differ from those liberated by the bacterium *in vivo*. Such a phenomenon was observed by Zhu *et al.*⁽⁹⁾ who investigated secondary electrospray ionisation-mass spectrometry (SESI-MS) to fingerprint pathogens directly from the breath of mice with lung infections and who demonstrated that only 25-34% of peaks were shared between the *in vitro* and *in vivo* SESI-MS fingerprints. Shyre *et al.*⁽⁶¹⁾ detected only one of four major biomarkers of *M. tuberculosis* (that were present *in vitro*) in the breaths of infected subjects. Additionally, genomic variation between strains could result in different efficiency of enzymes within particular metabolic pathways resulting in differing amounts of a particular VOC being generated.⁽⁸⁾

Finally, in other studies, smokers were specifically excluded as this is an obvious source of a heavy load of extraneous volatile compounds that could interfere with the detection of VOCs produced by *M. tuberculosis*.⁽⁷²⁾ Scott-Thomas *et al.*⁽⁷²⁾ showed that methyl nicotinate was isolated from the breaths of smokers making it unsuitable as a marker for TB. We wanted to test the sensor under real-world conditions where a significant number of the population smoke cigarettes. A number of participants in our trial smoked cigarettes (31%), regularly drank alcohol (33.6%), and admitted to utilising inhaled illicit drugs (8.6%). Interfering VOCs from these compounds still persisting in a participant's breath may be another contributing factor to the lack of specificity of the sensor.

6. LIMITATIONS

A limitation of the specific chemiresistive testing device used in our study is the number of sensors that could be fitted into the device whilst still maintaining portability of the device. Limiting the number of sensors compromises the number of compounds that can be detected as well as the discriminatory power of the sensor. Our test device contained 16 sensors. Limiting the number of sensors used in our device may have potentially impacted on the sensitivity and specificity of the device.

An additional limitation is that we lacked the specialised statistical software and experience of a statistician skilled in principal component analysis, linear discriminant analysis and fuzzy logic. Additional multivariate analysis of the data captured by the sensors may reveal whether factors such as HIV status, smoking or alcohol use are potential confounders in our study.

A major problem with conducting studies in this field are that there are different sensors on the market each detecting different chemical signatures using different technologies conducted in different settings in different patient populations where factors such as smoking or diet may impact on the results. This makes it difficult to replicate the findings of such studies. In their systematic review, Bos *et al.*⁽⁸⁾ found that some studies reporting on VOC production in pathogens implicated in sepsis failed to replicate the results of previous experiments producing contradictory results overall. The authors concluded that the conflicting results may be explained by four variables: genomic variation in subtype of bacterium resulting in differing production of VOCs; differing growth media which acts a source of

compounds for VOC generation; different VOCs being generated at different time points in the growth of a bacterium; and lastly, that most of the studies are based on cultures of reference strains and that clinical strains might differ in their generation of VOCs.⁽⁸⁾

Participants on treatment were not included in the study. Some studies have shown that the production of VOCs is decreased after the addition of antibiotics to culture media in levels greater than the minimal inhibitory concentration of the bacterium suggesting that potentially VOCs could be used to monitor response to therapy.⁽⁸⁾ This is an aspect that needs to be explored in future studies.

No patients with purely extrapulmonary tuberculosis were recruited for this study. Additional studies are recommended to determine if TB VOCs are generated in the breath of these patients and whether these VOCs can be detected via breath testing.

7. CONCLUSIONS

A future breath test could be a useful screening test for TB, particularly in active case-finding programmes. Our device shows some promise as a rapid, sensitive, cost-effective POC diagnostic for PTB. Whilst the sensor was able to discriminate between healthy controls and participants with culture/Xpert®-confirmed TB with a high sensitivity and specificity, it did not perform as well in distinguishing the culture-negative participants from culture-positive participants. Further research is still required to improve the

sensitivity and specificity of the device by choosing more selective sensors and VOC targets.

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APPENDIX

Appendix 1. Informed consent form: healthy controls.

Appendix 2. Informed consent form: participants suspected of having TB.

Appendix 3. Clinical research forms.

Appendix 4. Clinical research form: Patient follow-up.

Appendix 1. Informed consent form: healthy controls (English version).

INFORMED CONSENT FORM: Healthy Controls

Study Title: *Detection of Tuberculosis Using Chemiresistive Sensor Arrays*

Dear Sir/Madam,

The problem with TB tests is that all the tests that are available for doctors in the clinics to use at the moment are not completely accurate for diagnosing TB. What this means is that some people that actually have TB will be missed by doctors and will not be started on medication to fight their TB as early as possible.

For this specific reason, the University of Cape Town (Professor Mark Nicol from the Division of Medical Microbiology) is doing research on new TB tests. We need to compare the test in healthy people who do not have TB (such as you) with those who have suspected TB. We want to ask you, as a healthy volunteer, if you would be willing to be part of this study on TB. To be part of this study, you must be older than 18 years. If you agree to be part of the study, we will ask you to breathe into a small machine three times. We will wait 5 minutes between each breath, so the testing will take about 15 minutes. We will ask you not to smoke or eat anything for at least 2 hours before the test, since this could interfere with the test.

There are no risks to you if you decide to take part in this study. There is no chance that you could catch any disease from the test machine, since a completely new test device is used for each patient. Please, however, realise that the results of the tests that we do on you will not help you in any way – but what we eventually learn when the whole study is finished might help other people in the future. You will be given R50 for the time that you have used taking part in the study.

Throughout the study your privacy will be maintained. Nobody other than the study doctors and nurses will know that you are taking part in this study. Even when the study is finished, no person's name will be shown in publications.

The decision to participate is entirely your own. If you decide not to participate in the study this will not affect your employment in any way. It is completely up to you if, at any point, you choose to tell us that you do not want to be part of the study anymore. If you do withdraw from the study, at any point, this will not in any way have any effect on your employment.

This study will be monitored by the Research Ethics Committee of the University of Cape Town. During the study you may contact either the UCT Research Ethics Committee (021 406 6492) or the principal investigator (021 406 6083) if you have further questions.

Please ask the study researcher now if you have any questions about the study or about your decision to be involved or not. If you have decided to be part of the study, please fill in your name, your signature and the date below.

I, _____, have understood the above, and I have had the opportunity to discuss the study with the study researcher and ask any questions that I have. I consent to participate in this study:

Signature _____

Date _____

Name of study personnel taking consent: _____

Signature _____

Date _____

Name of the Witness: _____

Signature _____

Date _____

Appendix 2. Informed consent form: participants suspected of having TB (English version).

INFORMED CONSENT FORM: PATIENTS

Study Title: Detection of Tuberculosis Using Chemiresistive Sensor Arrays

Dear Sir/Madam,

You have been told by your clinic doctor that you may have tuberculosis (TB) and that you will have to have some tests done to try to see if you really have TB or not. The problem with these tests is that all the tests that are available for doctors in the clinics to use at the moment are not completely accurate for diagnosing TB. What this means is that some people that actually have TB will be missed by doctors.

For this specific reason, the University of Cape Town (Professor Mark Nicol from the Division of Medical Microbiology) is doing research on new TB tests. We want to ask you if you would be willing to be part of this study on TB. To be part of this study, you must be older than 18 years. If you agree to be part of the study, we will ask you to breathe into a small machine three times. We will wait 5 minutes between each breath, so the testing will take about 15 minutes. We will ask you not to smoke or eat anything for at least 2 hours before the test, since this could interfere with the test.

There are no risks to you if you decide to take part in this study. There is not chance that you could catch any disease from the test machine, since a completely new machine is used for each patient. The results of the tests that we do on you will not help you in any way – but what we eventually learn when the whole study is finished might help other people in the future. You will be given R50 for the time that you have used taking part in the study.

Throughout the study your privacy will be maintained. Nobody other than the study doctors and nurses will know that you are taking part in this study. None of the results from this study will be given to your clinic doctors or nurses who are treating you. Even when the study is finished, no person's name will be shown in publications.

The decision to participate is entirely your own. If you decide not to participate in the study this will not disadvantage your treatment in any way. It is completely up to you if, at any point, you choose to tell us that you do not want to be part of the study anymore. If you do withdraw from the study, at any point, this will not in any way have an effect on the treatment you receive from your clinic doctors or nurses.

This study will be monitored by the Research Ethics Committee of the University of Cape Town. During the study you may contact either the UCT Research Ethics Committee (021 406 6492), or the principal investigator (021 406 6083) if you have further questions. Please remember that these people are however not directly responsible for your medical care, which is the job of your clinic doctors and nurses.

Please ask the study researcher now if you have any questions about the study, or about your decision to be involved or not. If you have decided to be part of the study, please fill in your name, your signature and the date below.

I, _____ have understood the above, and I have had the opportunity to discuss the study with the study researcher and ask any questions that I have. I consent to participate in this study:

Signature _____ Date _____

Name of study personnel taking consent: _____

Signature _____ Date _____

Name of the Witness: _____

Signature _____ Date _____

[DATA COLLECTION SHEET]

Version 2

DIVISION OF MEDICAL MICROBIOLOGY
UNIVERSITY OF CAPE TOWN

DATA COLLECTION SHEET

CHEMIREISTIVE SENSOR ARRAY STUDY

Investigators: Prof Mark Nicol, Dr Mischka Moodley, Dr William Royea.

PATIENT NAME _____

FOLDER NUMBER _____

CHEMIREISTIVE SENSOR ARRAY STUDY

PT DETAILS

Surname _____ First names _____

Clinic _____ Clinic Folder number _____

Age _____ Gender _____

Address _____

Contact Telephone Number _____

MEDICAL HISTORY

Current Symptoms: Does the patient have any of the following symptoms. Please tick the appropriate box.

	Yes	No
Cough. How long? _____	☐	☐
Sputum produced	☐	☐
Night sweats	☐	☐
Haemoptysis (Coughing blood)	☐	☐
Fever	☐	☐
Difficulty breathing	☐	☐
Feeling tired easily	☐	☐
Other. If yes, please list _____	☐	☐

Past medical history:

Previous history of TB:

Yes ☐ No ☐

If yes, when? _____

Did they complete treatment?

Yes ☐ No ☐

How long was the treatment? _____ months

PATIENT NAME _____

FOLDER NUMBER _____

CHEMIREISISTIVE SENSOR ARRAY STUDY

Any recent TB contacts (family/ friends/ colleagues with TB in the last 6 months)?

Yes ☐ No ☐

Details : _____

Social History:

Does the patient smoke cigarettes or cigars? Yes ☐ No ☐

If yes, how many? _____ cig/day

Does the patient drink alcohol? Yes ☐ No ☐

If yes, how much? _____ units/wk (1 unit= 1 tot spirits, 1 glass wine or 1 can of beer)

Any other drugs used? Yes ☐ No ☐

Specify type of drug and frequency of use _____

OTHER INFORMATION

Did the patient have an HIV test done? Yes ☐ No ☐

What was the result? _____

Date test was performed: _____

Has the patient given informed consent? Attach consent form.

☐ Yes ☐ No

Has the patient eaten, drank fluids or smoked 2 hours before the test?

☐ Yes ☐ No

Breath sampling

The patient will take in a deep breath and then breathe out fully into the small container. Attach the container immediately to the machine. The machine will take 5 minutes to give a result. Perform the test three times.

	File name of results captured on computer
Test 1	
Test 2	
Test 3	

PATIENT NAME _____

FOLDER NUMBER _____

CHEMIREISTIVE SENSOR ARRAY STUDY

OTHER RESULTS:

Sputum microscopy Date sputa collected: _____

Smear 1 result :

☐ Smear positive ☐ Smear negative ☐ Not done. If not done, state reason _____

Smear 2 result :

☐ Smear positive ☐ Smear negative ☐ Not done. If not done, state reason _____

TB culture Date of sputum collection: _____

RESULT: _____

GeneXpert result Date of sputum collection: _____

☐ MTB detected ☐ MTB not detected ☐ No Result. Reason _____

HIV result ☐ Positive ☐ Negative

CD₄ count: _____ Date of CD₄ count: _____

Is patient on ARVs? Yes ☐ No ☐

CONTACT DETAILS OF INVESTIGATORS

Dr Mischka Moodley Email: mischka1@yahoo.com

Tel no: 021 406 6274

Prof Mark Nicol Email: mark.nicol@uct.ac.za

Tel no: 021 406 6083

Appendix 4. Clinical research form: Patient follow-up.

Chemiresistive Study : Patient Follow-Up

PATIENT DETAILS

Name _____ Folder number _____

MEDICAL HISTORY

Current Symptoms: Does the patient have any of the following symptoms. Please tick the appropriate box.

	Yes	No
Cough. How long? _____	☐	☐
Sputum produced	☐	☐
Night sweats	☐	☐
Haemoptysis (Coughing blood)	☐	☐
Fever	☐	☐
Difficulty breathing	☐	☐
Feeling tired easily	☐	☐
Other. If yes, please list _____	☐	☐

Did the patient receive any treatment? Yes ☐ No ☐

If yes, what was the treatment _____ -

Did the patient receive any anti-TB medication? Yes ☐ No ☐

Does the patient feel better now? Yes ☐ No ☐

Pt meets inclusion criteria Yes ☐ No ☐

CHAPTER THREE: FEASIBILITY OF USING THE DETERMINE™ LATERAL FLOW LIPOARABINOMANNAN TEST FOR THE DIAGNOSIS OF TUBERCULOSIS (TB) IN HIV/TB CO-INFECTED PARTICIPANTS IN CAPE TOWN, SOUTH AFRICA

I. INTRODUCTION

A true point-of-care (POC) test has remained elusive for the diagnosis of TB. Such tests have transformed the diagnosis of other infectious diseases, such as malaria, cryptococcal meningitis and HIV.^(1, 2) These tests have had a major impact in low-resource settings where access to healthcare and laboratory diagnostics is limited. POC tests offer rapid results allowing for earlier diagnosis and initiation of life-saving therapy.

Smear microscopy (the mainstay of TB diagnosis in many middle- and low-income countries) is simple, rapid and inexpensive with limited infrastructure required for testing.^(3, 4) One of the major limitations of the test however has been the lack of sensitivity, particularly when the bacterial load is less than 10,000 organisms per millilitre of sputum.⁽⁵⁾ The test has been shown to perform poorly in paediatric and HIV-infected patients, and in cases of extrapulmonary TB.⁽⁶⁻¹⁰⁾ Sputum is not always an easily obtainable specimen. Lawn *et al.*⁽¹¹⁾ found that only 37% of hospitalised HIV-infected patients could produce a sputum specimen within 24 hours of admission despite careful instruction, assistance from a study nurse and the use of nebulised induction. Another disadvantage is that in the ambulatory setting,

patients often don't return for serial sputum examinations, or to receive their results.⁽¹²⁾

Nucleic acid amplification tests (NAATs), such as the Xpert®MTB/RIF assay (Cepheid, Sunnyvale, California, USA) have revolutionised TB diagnosis. These tests are able to be performed close to or at the POC permitting rapid diagnosis within hours after specimen receipt. NAATs are more sensitive than smear microscopy⁽¹³⁻¹⁵⁾ and also provide genotypic drug susceptibility results enabling rapid initiation of appropriate therapy as well as infection control procedures to be implemented. The disadvantage is that such technology is expensive, requires delicate equipment, and a stable electrical supply. Whilst these tests represent a significant improvement on previous diagnostics, they remain unavailable to the majority of patients in low- and middle-income countries where the burden of TB remains the highest.^(16, 17)

A true POC test for TB should be performed close to the patient, is cost-effective, easy to perform and interpret. It should not require laboratory infrastructure and ideally be suitable for use in areas where there is no cold chain or uninterrupted electricity supply. Such a test will improve the standard of care by reducing the number of patients lost to follow-up and obviate the need to refer specimens from rural areas to centralised laboratories in the cities. It will reduce unnecessary work in the laboratory whilst at the same time streamlining care in the clinical setting by enabling larger volumes of patients to be tested. Based on mathematical modelling, this ideal TB test (assuming a 100% sensitivity and specificity) could reduce mortality due to TB by up to 625,000 lives or 36% of the total TB deaths each year.⁽¹⁸⁾

Urine antigen-based testing for diagnosis of TB is an attractive format for POC testing. The advantages of such a test are that the specimen is easily collected (non-invasive), poses less of an infectious risk to healthcare workers (HCWs) and laboratory workers, the test is likely to be relatively inexpensive and could be performed at the site of patient contact. In addition, as HCWs are familiar with reading urine test strips for other clinical conditions, for example pregnancy tests, it would be relatively easy to implement such a test which could be performed rapidly and read by staff at the point of contact obviating the need for laboratory infrastructure. Although such a test format would likely not yield drug susceptibility testing (DST) results, it could be used as a screening test and thus enable resources to be diverted to additional testing for culture and DST for patients who test positive by screening. This will reduce the economic as well as laboratory burden of unnecessary TB culture or molecular testing.

The lipoarabinomannan (LAM) antigen, a component of the cell wall of *Mycobacterium tuberculosis*, is a possible candidate for such a test. LAM is a mannose-containing glycolipid which is an important component of the mycobacterial cell wall.⁽¹⁹⁾ This molecule has a phosphatidyl-inositol (PI) membrane anchor and is embedded in the plasma or cell membrane by its lipid moiety⁽¹⁹⁾ [Figure 1]. LAM is synthesized by sequential addition of mannoses and arabinoses to PI.⁽¹⁹⁾ This cell wall component is found amongst the mycobacteria, but also amongst closely related bacteria, such as *Corynebacterium* spp.⁽²⁰⁾, *Rhodococcus* spp.⁽²¹⁾, *Tsukamurella* spp.⁽²²⁾, *Turicella* spp.⁽²³⁾, *Amycolatopsis* spp.⁽²⁴⁾ and *Saccharothrix* spp.⁽²⁵⁾.

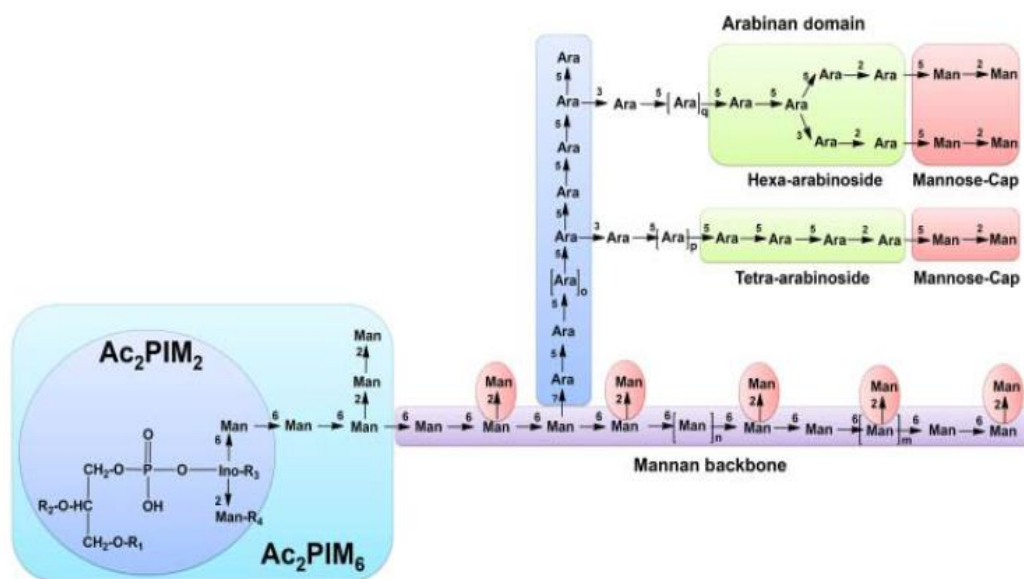


Figure 1. Schematic structure of LAM and related glycoconjugates.⁽²⁶⁾

Reproduced from: Mishra AK, Driessen NN, Appelmelk BJ, Besra GS; Lipoarabinomannan and related glycoconjugates: structure, biogenesis and role in *Mycobacterium tuberculosis* physiology and host-pathogen interaction; FEMS Microbiol Rev; 2011;35(6); pages 1126-1157; by permission of Oxford University Press and the Federation of European Microbiological Societies.

Key: Ac₂PIM₂ tetra-acylated phosphatidyl-*myo*-inositol-dimannoside, Ac₂PIM₆ tetra-acylated phosphatidyl-*myo*-inositol-hexamannoside, Man mannose, Ara arabinose.

LAM plays an important role as a virulence factor for *M. tuberculosis*. ManLAM (mannose-capped LAM) which is a Pathogen-Associated Molecular Pattern (PAMP) displays immunomodulatory effects by interacting with receptors of the immune system, such as C-type lectin Mannose Receptor, and thus subverting the immune response.^(27, 28) In addition, LAM functions to protect the bacterium at multiple levels, by scavenging potential free oxygen radicals, inhibiting protein kinase C activity, blocking the transcriptional activation of gamma interferon-inducible genes in human macrophages, inhibiting phagolysosome biogenesis, and inhibiting apoptosis as well as autophagy.^(28, 29) It is a highly immunogenic antigen⁽³⁰⁾ and anti-LAM antibodies have been identified in a

variety of clinical specimens from TB-infected patients, including sputum and pleural fluids.⁽³¹⁾ However, as antibody responses are variable in infected subjects, the 2011 policy statement by the World Health Organisation (WHO) concluded that TB serological tests detecting anti-LAM antibodies should not be used for diagnosis.⁽³²⁾

The LAM antigen itself may also be used as a possible diagnostic target for development of rapid POC testing. This 17.5 kilodalton heat-stable cell wall component is released from active or degrading *M. tuberculosis* cells.⁽³³⁾ It is produced in large quantities by the bacterium.⁽³⁴⁾ LAM production appears to be strain-dependent, and the volume of LAM produced is dependent on the bacterial load.⁽³⁵⁾ It is hypothesized that LAM is secreted by infected alveolar macrophages and that high tissue concentrations of the antigen may favour its entry into the systemic circulation.^(36, 37) Theoretical advantages of detection of LAM antigen include quantification of organism load as well as applicability in diagnosis of extrapulmonary TB. LAM has been detected in various specimen types from patients including blood^(31, 37), urine⁽³⁸⁾, cerebrospinal fluid^(39, 40) and sputum⁽⁴¹⁾.

The exact mechanism whereby LAM enters the urine is uncertain. Some authors have postulated that LAM is secreted by *M. tuberculosis* during active infection and filtered by the kidneys into the urine, thus forming the basis for the urine LAM assays.^(42, 43) Another hypothesis is that the urine LAM test detects active TB infection of the renal tract.⁽⁴³⁾ Two studies^(43, 44) have demonstrated that approximately half of the participants with a positive urine LAM lateral flow assay (LFA) had a positive Xpert®MTB/RIF assay on urine. As the Xpert® assay detects whole bacilli; a positive test result indicates the presence of bacteria within the renal tract likely representing renal tract disease. A post-mortem study⁽⁴⁵⁾ performed in Uganda found that eight of 13 individuals with a positive urine LAM test had histological

abnormalities consistent with renal TB. Of the 23 participants with a negative urine LAM test, none had histological evidence compatible with renal TB.⁽⁴⁵⁾ It is likely that both mechanisms (free unbound LAM passing into the urine and renal tract TB) account for positive urine LAM tests. The excretion of LAM in urine may be independent of the site of the primary infection, and thus potentially could serve as a useful test for all forms of TB.

In an early report, Sada *et al.*⁽³⁷⁾ reported a sensitivity of 88% and 67% in patients with smear-positive TB and smear-negative TB respectively when an in-house LAM enzyme linked immunosorbant assay (ELISA) was performed on blood specimens. In patients with AIDS and TB, the sensitivity dropped to 57%.⁽³⁷⁾ There are also reports of false negative blood LAM tests due to the formation of immune complexes as a result of high levels of antibodies in patients with TB.⁽³¹⁾ To circumvent this, assays detecting LAM antigen in blood often include a dissociation step.⁽⁴⁶⁾ Sakamuri *et al.*⁽⁴⁷⁾ demonstrated that LAM is associated with lipoprotein carriers such as high density lipoprotein in blood, and this may account for low levels of free monomeric LAM in blood, and hence false negative LAM tests.

The detection of LAM in sputum may be useful for patients with pulmonary TB (PTB). Dheda *et al.*⁽⁴⁸⁾ found in their cohort study of 377 HIV-infected individuals in South Africa that sputum-LAM ELISA had good sensitivity (86%; 95% confidence interval (CI) [81,90]) but poor specificity (15%; 95% CI [10, 21]) likely due to test cross-reactivity with several members of the oral flora such as yeasts, actinomycetes and *Nocardia* species. Peter *et al.*⁽⁴⁹⁾ reported a sensitivity of the LAM ELISA of 56% (95% CI [27, 81]) and a specificity of 48% (95% CI [34, 62]) in their study on induced sputa from 53 participants with suspected TB that presented to a respiratory clinic at a tertiary level hospital in South Africa.

Hamasur *et al.* showed that LAM was detectable in urine using a murine model as well as in patients with active TB.⁽³⁸⁾ Commercial ELISAs for detection of LAM from urine have been available for a number of years.^(42, 48, 50, 51) Early developmental versions of these assays showed moderate sensitivity and specificity and required extensive urine purification that included column chromatography.^(31, 38) These formats were impractical as simple POC diagnostic tests for resource-limited environments. Following these early developmental studies of urine ELISA and lateral flow testing by Hamasur *et al.*⁽³⁸⁾, a first generation prototype MTB LAM ELISA (Chemogen, Portland, USA) was trialled⁽⁴²⁾ A second generation assay followed using a different set of polyclonal antibodies, which was in turn replaced by a commercial assay [Clearview TB ELISA, Alere Inc. (formerly Inverness Medical Innovations Inc.), Waltham, Massachusetts, USA] which uses the same polyclonal antibody as the Chemogen assay.⁽⁵²⁾ The ELISA is usually performed in a 96-well plate which passively binds antigens between two layers of antibodies, the capture and detection antibodies (Figure 2).

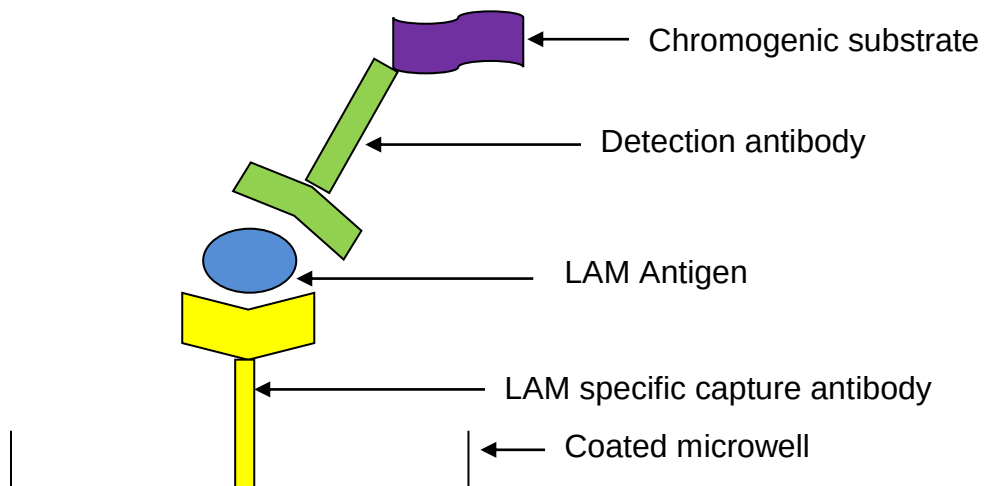


Figure 2. Illustration depicting the direct antigen sandwich ELISA.

The ELISA test requires specimen preparation and batch processing which is often performed in central laboratories. This format is not feasible as a POC test in resource-limited settings. The Determine™ TB-LAM Ag test (Alere, Waltham, Massachusetts, USA) is an “immunochromatographic test in which the capture antibodies are adsorbed onto a nitrocellulose membrane of the test strip” [Figure 3].⁽³³⁾ The detection antibody is labelled by conjugation to colloidal gold particles.⁽³³⁾ The gold particles in the sample pad bind to LAM present in the patient’s urine sample.⁽³³⁾ As the patient’s urine moves along the test strip, the urine LAM is captured by the LAM capture antibodies on the nitrocellulose membrane.⁽³³⁾ The gold particles result in the development of a purple line in the test strip, indicating a positive result.⁽³³⁾ The intensity of the purple line is then compared to the reference card provided by the manufacturer.⁽³³⁾ The test is read by visual inspection within 25 minutes and requires no additional processing of the urine specimen, equipment or supplies.⁽³³⁾ The Determine™ LAM lateral flow assay (LFA) has a shelf-life of nine months and can be stored between 2-30°C.⁽³³⁾

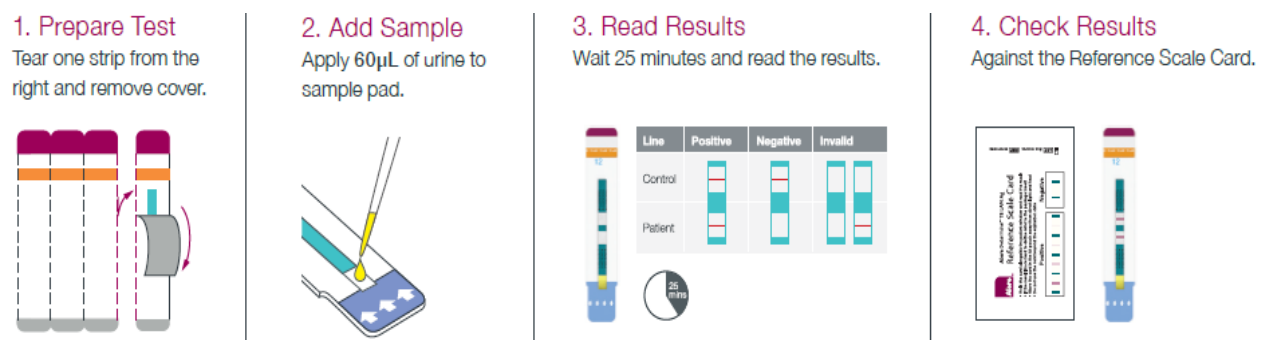


Figure 3. Test methodology for the lateral flow Determine™ TB-LAM Ag test.⁽³³⁾

Several studies evaluating the accuracy of the urine LAM test (ELISA and/or LFA) for diagnosis of TB have been performed. The sensitivity of the assays on urine varies from 21% to 88% in HIV-infected individuals and 6% to 74%

in HIV-uninfected patients with suspected TB.^(42, 50, 53-57) A recent meta-analysis⁽⁵²⁾ of studies using commercial LAM assays on urine from patients with microbiologically confirmed TB reported sensitivities of 13-93% and specificities of 87-99%. Of note, sensitivity was increased in HIV co-infected individuals and was highest in those with greater immunosuppression.⁽⁵²⁾ Another systematic review and meta-analysis⁽⁵⁸⁾ confirmed that the “pooled sensitivity of urine LAM was higher in HIV-infected than HIV-uninfected individuals: 47% (95% CI [26, 68]) versus 14% (95% CI [4, 38])”. Pooled specificity estimates were similar: 96% (95% CI [81,100]) and 97% (95% CI [86, 100]) in HIV-infected and HIV-uninfected patients, respectively.⁽⁵⁸⁾ Wood *et al.* reported the sensitivity of LAM LFA in HIV-infected patients ($n=146$) with CD4 T-cell counts of ≥ 200 , 100-199, 50-99, and <50 cells/ μl , was 15.2%, 32%, 42.9%, and 69.2% respectively.⁽⁴³⁾ Shah *et al.*⁽⁵¹⁾ reported LAM sensitivity was 55% (95% CI [41, 69]) for those with CD4 T-cell counts greater than 200 cells/ mm^3 , 14% (95% CI [3.6, 58]) for CD4 counts of 150–200 cells/ mm^3 , 56% (95% CI [30, 80]) for CD4 counts of 100–150 cells/ mm^3 , 71% (95% CI [51, 87]) for CD4 counts of 50–100 cells/ mm^3 , and 85% (95% CI [73, 93]) for CD4 counts less than 50 cells/ mm^3 . The utility of this test as a screening test for TB in the ambulatory outpatient setting appears limited.^(52, 59) A recent study⁽⁶⁰⁾ in the paediatric population revealed a sensitivity of 48.3% (95% CI [37.6, 59.2]), and a specificity of 60.8% (95% CI [56.1, 65.3]) when compared to TB culture.

Although the underlying reasons for the wide discrepancy in performance is not entirely understood, it may possibly be due to multiple factors: different assay formats (ELISA versus lateral flow testing), different manufacturers, lack of standardisation of the polyclonal antibody preparations, batch variations between different lots of test kits, lack of standardisation of investigation of TB by investigators, diversity of patient selection (inclusion of HIV-uninfected as well as HIV-infected participants), and testing on frozen specimens in certain studies. Other authors have speculated that

storage of urine in non-sterile containers and the frequency of non-tuberculous mycobacteria in the various studies could also account for the discrepancy.⁽⁶¹⁾ Differences in interpretation of positive cut points on the LFA strip (grade 1 versus grade 2) may also account for differences noted.

As there is significant interest in the use of such a test as a POC test, we sought to perform a large definitive evaluation of the lateral flow and ELISA urine LAM assays by performing a multicentre prospective study in Cape Town, South Africa and Kampala, Uganda, to evaluate the true utility of this test in the context of HIV and TB co-infection as well as to detect possible underlying causes for the variability of sensitivity and specificity reported in the literature to date. We compared the lateral flow Determine™ TB-LAM Ag test to Clearview™ urine LAM ELISA and the reference standard of liquid mycobacterial culture of sputum and blood. Only the results from the Cape Town cohort will be described further, since these formed part of the candidate's PhD.

2. AIMS

2.1 To determine the sensitivity and specificity of the urine LAM LFA (Determine™ TB-LAM Ag test) in HIV-infected adults with clinical signs and/or symptoms of TB in comparison to the reference standard of liquid mycobacterial culture of sputum and/or blood.

2.2 To compare the sensitivity and specificity of the urine LAM LFA (Determine™ TB-LAM Ag test) against the Clearview™ TB ELISA (Alere, Waltham, MA, USA).

2.3 To determine the clinical characteristics associated with a positive urine LAM LFA test.

2.4 To determine if there is any correlation between a positive Determine™ TB-LAM Ag test on urine specimens and CD4 T-cell count.

3. METHODOLOGY

This prospective cross-sectional diagnostic accuracy study was performed at two sites in Cape Town, South Africa, to evaluate the utility of the test in both the ambulatory (outpatient) as well as hospitalised patient settings. There was limited longitudinal follow-up of participants meeting certain predefined criteria which is described below.

3.1 Clinical sites

Participants were recruited from both hospitalised (GF Jooste Hospital) and outpatient (Town Two clinic, Khayelitsha) settings in Cape Town, South Africa. GF Jooste is a district level hospital with specialist physician services available. Town Two clinic is a primary care clinic with dedicated HIV/TB services. The number of new cases of TB in 2010 in the Western Cape was 49,819 cases (incidence rate of 885/100,000 population) and the prevalence of HIV in the Cape metropole as per the 2010 antenatal survey was 20.2% (95% CI [15.7,25.6]).⁽⁶²⁾ Participants were enrolled between January 2011 and November 2011. All study procedures were performed according to the study protocol as well as a written procedures manual.

3.2 Participants

3.2.1 Inclusion criteria

In order to be eligible for the study, participants had to fulfil the following inclusion criteria:

- age ≥ 18 years;
- provide informed consent;
- have suspected active TB with any one or more of the following symptoms:
 - cough;
 - fever at any time within the preceding four weeks;
 - night sweats at any time within the preceding four weeks; and/or
 - weight loss within the preceding four weeks;
- HIV-infected, based on any one or more of the following: written results of a positive HIV antibody test and/or written results of a positive HIV viral load and/or documentation in the medical record of positive HIV status by a treating clinician; and
- willingness and ability to comply with study procedures.

3.2.2 Exclusion criteria

Participants fulfilling the following criteria were excluded from the study:

- age < 18 years;

- HIV-uninfected or HIV status unknown;
- taking TB treatment for longer than two days within the previous 60 days;
- unwillingness or inability to provide a urine sample;
- known chronic pulmonary condition, e.g., asthma, chronic obstructive pulmonary disease, emphysema;
- respiratory distress, defined as respiratory rate of >30 breaths per minute or oxygen saturation <90%; or
- any specific condition that in the judgment of the investigator precluded participation because it could affect a subject's safety.

3.3 Study flow

3.3.1 Enrolment/ baseline visit

The following study evaluations were performed at the time of enrolment (Figure 4):

- Targeted medical history.
- Targeted medication history focusing on prescription medications for TB and HIV.
- Weight.
- Karnofsky score.
- Two sputum specimens were collected for mycobacterial microscopy and culture. The sputa were obtained at the same visit, with a minimum window of 60 minutes between sputum collections. The allowable maximum window was four days.

Participants unable to provide any spontaneously expectorated sputum specimen underwent one sputum induction. Sputum induction was performed according to a written procedure, and included pre- and post-procedure assessments for safety.

- Blood (approximately 10 mL) was drawn for enumeration of CD4 T- cells.
- Blood (approximately 5 mL) was drawn for mycobacterial blood culture.
- Urine (a minimum of approximately 20 mL) was obtained and utilised for the Determine™ LAM LFA, the Clearview™ TB ELISA assay, as well as routine chemical dipstick analysis.
- A pregnancy test was performed on all female participants, unless the participant was known to be pregnant or the written results of a negative test done within the preceding three days of the baseline visit was available. This was performed to ensure that pregnant participants did not undergo a chest radiograph (CXR).
- Chest radiograph for males and non-pregnant females. The CXR was categorised as likely TB, probable TB or not TB by either a consultant radiologist (outpatient CXR) or the study medical officer (hospitalised patients).

3.3.2 Follow-up visit

Participants who at baseline had any positive urine LAM test but no positive mycobacterial culture for *M. tuberculosis* had a follow-up study visit to establish a diagnosis with respect to TB. The follow-up visit was performed approximately two months after enrollment (window period six to 16 weeks).

The following data/testing were obtained at follow-up:

- Targeted interval medical history including TB treatment.
- Weight.
- Karnofsky score.
- One sputum specimen was obtained for AFB smear microscopy and mycobacterial culture. Participants unable to provide any spontaneously expectorated sputum specimen underwent sputum induction. Sputum induction was performed according to a written procedure, and included pre- and post-procedure assessments for safety.
- Blood (approximately 10 mL) was drawn for mycobacterial blood culture.
- Urine (a minimum of approximately 20 mL) was obtained and utilized for the Determine™ LAM LFA, the Clearview™ TB ELISA assay, as well as routine chemical dipstick analysis.
- Pregnancy test for all female participants, unless the participant was known to be pregnant or the written results were available for a negative test done within the preceding three days of the follow-up visit.
- CXR for males and non-pregnant females. The CXR was categorised as likely TB, probable TB or not TB by either a consultant radiologist (outpatient CXR) or the study medical officer (hospitalised patients).

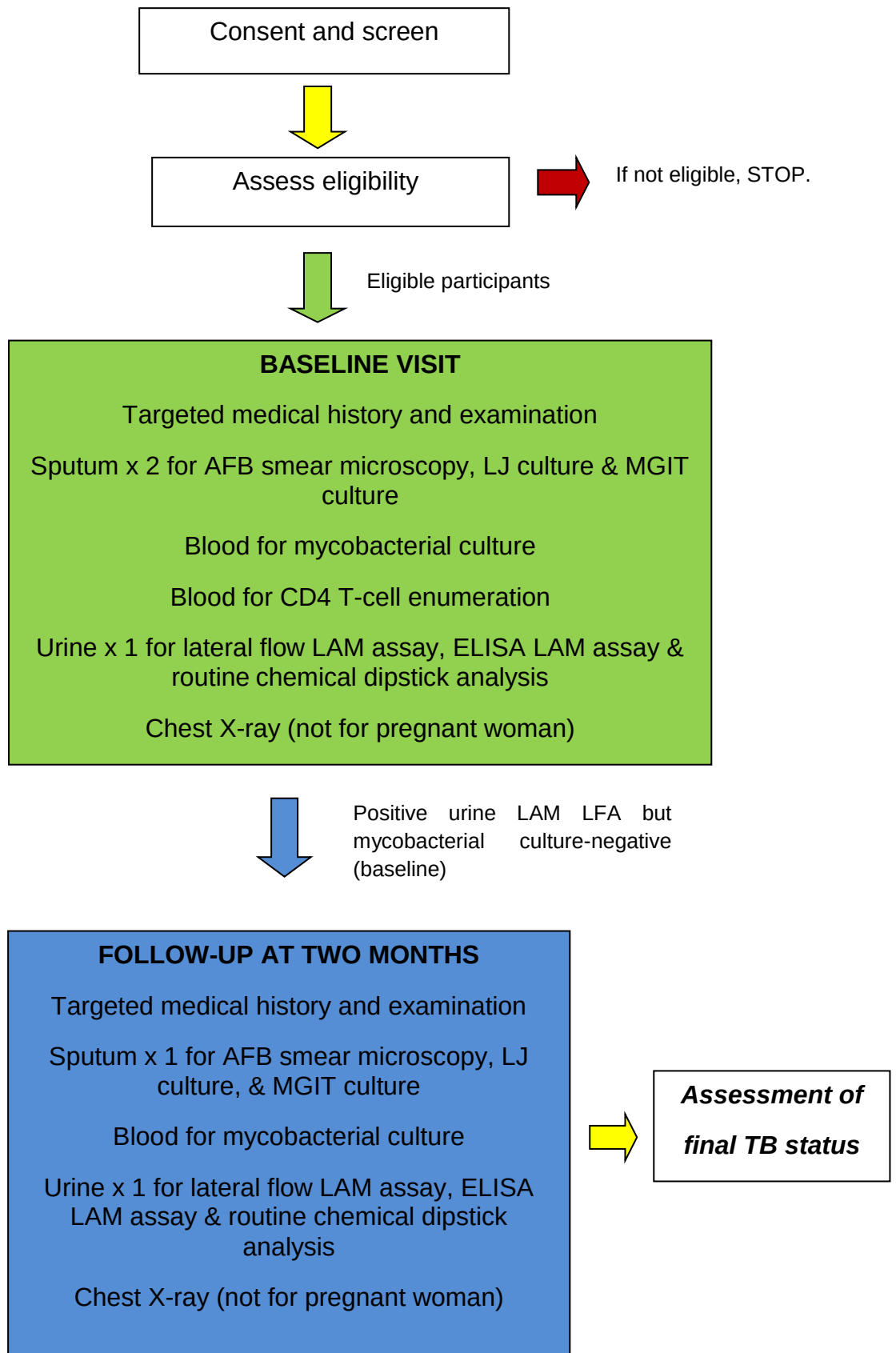


Figure 4. Schematic of study design.

3.4 Microbiological culture: sputum and blood

Direct as well as concentrated fluorescent acid fast bacilli (AFB) sputum smear microscopy (as per WHO guidelines), Mycobacterial Growth Indicator Tube (MGIT) culture (BACTEC MGIT 960, Becton Dickinson, and Company, New Jersey, USA) and Lowenstein Jensen (LJ) culture were performed on the N-acetylcysteine-sodium hydroxide-treated specimens at the National Health Laboratory Services (NHLS) Medical Microbiology Laboratory at Groote Schuur Hospital, Cape Town (a South African National Accreditation System accredited laboratory with external quality assurance programmes). The identification and susceptibility of cultured isolates were confirmed using the GenoType® MTBDR*plus* line probe assay (Hain Lifescience, GmbH, Nehren, Germany). Due to financial constraints, the Xpert MTB/RIF® assay was not performed on sputum. In addition, the Xpert® assay was not available as routine standard of care in South Africa at the time the study was conducted.⁽⁶³⁾

Approximately five millilitres of blood was collected in BacT/ALERT® MP mycobacterial blood culture bottles (bioMerieux, Marcy-l'Etoile, France) as per manufacturer's instruction. Bottles were incubated in a continuously monitored blood culture system (bacT/ALERT®3D Microbial Detection System, bioMerieux, Marcy-l'Etoile, France) at the NHLS laboratory. The identification and susceptibility of cultured isolates were confirmed using the GenoType® MTBDR*plus* line probe assay or in the event of duplicate isolates from the same participant, the anti-MPB 64 antibody assay (Capilia TB-Neo, TAUNS Laboratories, Numazu, Japan).

The results of positive microbiological cultures were relayed to the relevant physicians providing care to the participants.

3.5 Clearview™ TB ELISA

The assay was performed as per manufacturer's instructions. Briefly, one to two millilitres of fresh (< 24 hrs post collection) urine was heated between 95-100°C for 30 minutes using a boiling water bath. The sample was allowed to cool to room temperature and centrifuged for 15 minutes as per manufacturer's instructions. One hundred microlitres (µL) of the supernatant was pipetted into the microwell and incubated for 60 minutes at 20-25 °C. The plate then underwent four wash steps. Following the wash, 100 µL of the HRP-conjugate was added to each well. The plate was incubated again for 60 minutes at 20-25 °C. The contents of the well were decanted and the plate washed again for a total of four washes. One hundred microlitres of the chromogenic substrate solution (TMP) was added to each well. The plates were incubated again at 20-25 °C for 15 minutes, after which 100 µL of stop solution was added. The optical density was then read by a microtitre plate reader (Bio-Rad Laboratories Ltd., Johannesburg, South Africa) at 450 nm.

Results were calculated by averaging the duplicate optical density (OD) readings obtained for the controls as well as the participant samples. A participant sample was considered negative if the average participant sample OD was less than the positive/negative cut-off value. A participant sample was considered positive if the average participant sample OD was greater than or equal to the positive/negative cut-off value. The participant sample was invalid if the positive and negative controls were out of range. The test was repeated if an invalid result was obtained.

3.6 Determine™ TB-LAM Ag test

The test principle was described previously. As per manufacturer's instructions, a test strip was removed from its protective foil packaging, 60 µL of fresh unprocessed urine collected by midstream collection was then pipetted onto the sample pad of the strip, and the strip was left for 25 minutes prior to reading.⁽³³⁾

3.6.1 Reading of the strips

The strips were read as per manufacturer's instructions.⁽⁶⁴⁾ The presence of a purple/grey bar in the control window indicated that the test result was valid; whilst absence of a purple bar in the control window indicated that the test was invalid.⁽⁶⁴⁾ The test was repeated if invalid.

A positive result was obtained if a purple/grey bar was obtained in both the patient and control window [Figure 5].⁽⁶⁴⁾ Similarly, a negative result was obtained if a purple/grey bar was missing from the patient window, but was present in the control window.⁽⁶⁴⁾ An equivocal or indeterminate result was obtained if a purple/grey bar was present in the control well with an unclear or incomplete bar in the patient window.⁽⁶⁴⁾ The test was repeated in the event of an equivocal or indeterminate test result.

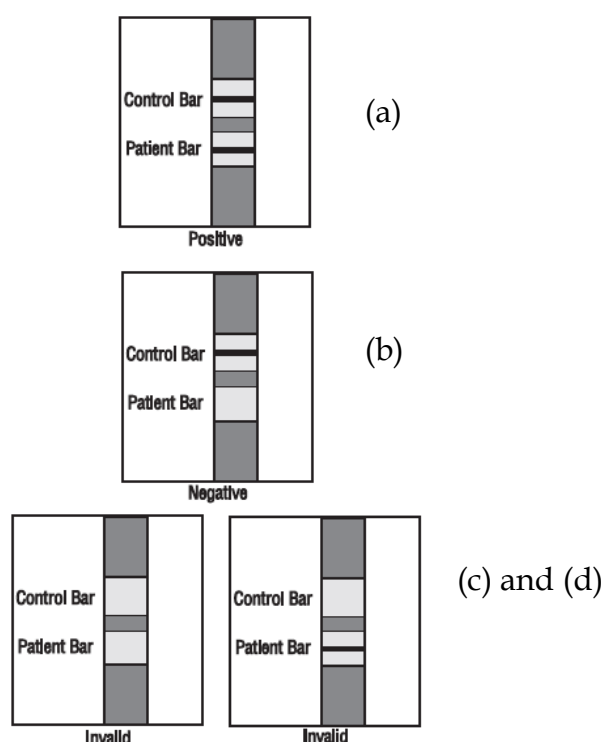


Figure 5. Depiction of various test results using the Determine™ TB-LAM Ag test.⁽⁶⁴⁾

Key: (a) indicates a positive result; (b) indicates a negative result; (c) and (d) indicate invalid results (control bar missing).

The strips were independently read by two trained study staff to observe for inter-reader correlation. Each reader was blinded to the result of the other reader as well as the results of clinical data or additional microbiological testing. The intensity of the band was compared to the reference scale card that was supplied by the manufacturer to determine if there was an optimal cut-off that would improve the sensitivity and specificity of the test. The intensity was graded a zero if there was an absence of a band through to grades one ($\pm$ on dipstick) through five (4+ on dipstick) for visualised bands. **For the purposes of this study, the reading by the first reader was used as the result of the study test. Unless otherwise stated, a band threshold positivity of grade 2 (1+ on dipstick) or higher was considered a positive result** (Figure 6). Prior studies had confirmed that a grade 2 cut-point was the optimal cut-point which resulted in the manufacturer modifying the

reference scale card in January 2014.^(61, 65) A late read performed 60-90 minutes after incubation (i.e. 35-65 minutes after the initial reading) by reader one was also performed. The result of the urine LAM LFA test was not communicated to the physician providing care to the participant and therefore was not used in the clinical decision making process.

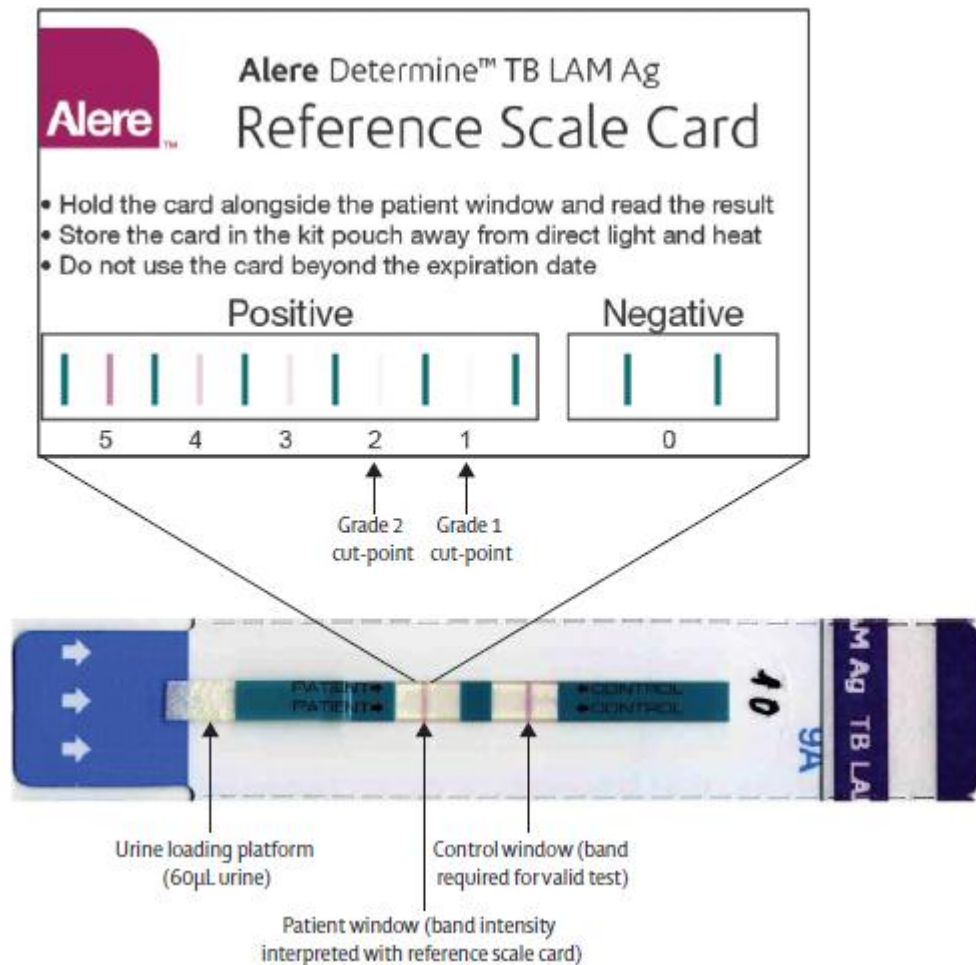


Figure 6. Alere Reference Scale Card for interpretation of band intensity.⁽⁶⁶⁾

The reference card shows six cut-points (visual grades 0-5) categorised by different band intensities in the patient window. [Reprinted from Lancet Infectious Diseases; Vol. 12; Issue 11; Peter JG, Theron G, Dheda K; Urine antigen test for diagnosis of HIV-associated tuberculosis; Page 825, Copyright 2012, with permission from Elsevier].

In addition, urine dipstick testing (Combur-Test; Roche, Basel, Switzerland) for detection of protein, glucose, leukocytes, nitrites, and erythrocytes was performed on all urine specimens collected.

3.7 Case definitions

Based on the results of microbiological testing, the participants were categorised into three groups ('Definite TB', 'Probable TB' and 'Not TB') according to strict definitions. The definitions were as follows:

- 'Definite TB' was based on *M. tuberculosis* complex being cultured from a clinical specimen (sputum and/or blood).
- 'Probable TB' was based on any one or more of the following: smear microscopy positive for AFB, but no culture positive for *M. tuberculosis* or other non-tuberculous mycobacteria; caseous necrosis or granulomas on histopathology (if done), and/or clinical response to TB treatment, absence of criteria for 'Definite TB' and absence of a plausible non-TB alternative diagnosis.
- Classification as 'Not TB' was based on the presence of a plausible alternative diagnosis and that the participant did not meet criteria for 'Definite TB' or 'Probable TB'.

As the intention was to investigate the sensitivity and specificity of the urine LAM LFA at the first visit at the point-of-care (as this is the envisioned use of the assay), **study follow-up data at two months (including microbiology culture results and record review data) was not included in the definition of 'Definite TB'**. Only one additional patient was confirmed to be culture-positive based on study microbiological culture results at two month follow-

up, hence the exclusion of this additional data did not have a significant impact on the current results.

3.8 Data collection, collation, statistical analysis

Our sample size calculations indicated that in order to obtain 100 culture-positive TB cases, with 95% confidence intervals (CIs) of $\pm 10\%$ over a range of investigational test sensitivities of 30-70%, a sample size of 500 participants was required (assuming 20% prevalence of culture-positive TB amongst participants).

All data was collected on standardised clinical research forms and captured onto a secure electronic database. The data system included password protection as well as internal and external quality control checks. Simple descriptive statistics were used to categorise the study population using Stata v12 software (Statacorp, College Station, Texas, USA). The kappa co-efficient was used to determine inter-reader correlation. For the primary analysis of the accuracy of the Determine™ LAM LFA and Clearview™ TB ELISA, positive mycobacterial culture (i.e. 'Definite TB') at baseline was used as the reference standard. Sensitivity, specificity, and predictive values of the assays with 95% CIs were also calculated. Univariate analysis was performed using the Pearson Chi-square test, Fisher's exact test or Mann-Whitney test as appropriate on SPSS statistical software (IBM SPSS Statistics for Windows, version 22.0, Armonk, New York, USA). As the number of patients with a positive urine LAM LFA test result ($n=58$) would only allow for a maximum of six clinical characteristics in the logistic regression model, purposeful selection of clinical characteristics with $p < 0.1$ at the univariate level was used to select potentially relevant characteristics for inclusion in the logistic regression analysis and subsequent calculation of adjusted odds ratios.⁽⁶⁷⁾

Receiver operator characteristic (ROC) analysis was performed to evaluate sensitivity and specificity at different band intensity thresholds of the urine LAM LFA using MedCalc for Windows, version 12.6.1 (MedCalc Software, Ostend, Belgium). The categories of 'Definite TB' and 'Not TB' were used to calculate the ROC curve ('Possible TB' category was excluded). Statistical tests were two-sided at α of 0.05.

3.9 Ethical approval

The study received ethics approval from the University of Cape Town Human Research Ethics Committee (HREC Ref 045/2008) as well as Johns Hopkins University School of Medicine Ethics Committee, Joint Clinical Research Centre (Kampala, Uganda) and Uganda National Council for Science and Technology. Approval from the City of Cape Town (Town Two Clinic) as well as the Western Cape Provincial Department of Health was obtained. All participants provided written informed consent prior to enrolment.

3.10 Clinical monitoring

Site monitoring was conducted to ensure that:

- participant rights and well-being were protected;
- data was accurate, complete, and verifiable from source documents; and
- the study complied with the protocol, Good Clinical Practice guidelines, and applicable regulatory requirements.

In order to ensure protocol compliance, monitoring visits by a sponsor-designated monitor occurred at scheduled intervals prior to, during, and at study completion. The visit frequency was defined in a monitoring plan and communicated before study start to the principal investigators and all other appropriate study personnel. Monitoring visits included, but were not limited to, review of regulatory files, clinical research forms, informed consent forms, medical and laboratory reports, and protocol compliance. Study monitors met with investigators to discuss any problems and actions to be taken and documented visit findings and discussions.

3.11 Funding source

The study was designed and implemented by investigators of the TB-Clinical Diagnostics Research Consortium (TB-CDRC). Neither the sponsor nor Alere were involved in study design, implementation, or analysis of the study.

4. RESULTS

4.1 Baseline characteristics

Five hundred and seven participants were enrolled in the study. Fourteen participants were excluded. Reasons for exclusion included: sputum specimens not provided by participants or collected within the designated time frame (six participants); two participants were found to be HIV-uninfected on subsequent testing; two participants were commenced on TB treatment prior to collection of blood cultures; one participant died prior to collection of specimens and three participants were duplicated in error. Four

hundred and ninety-three participants were included in the final analysis (Figure 7).

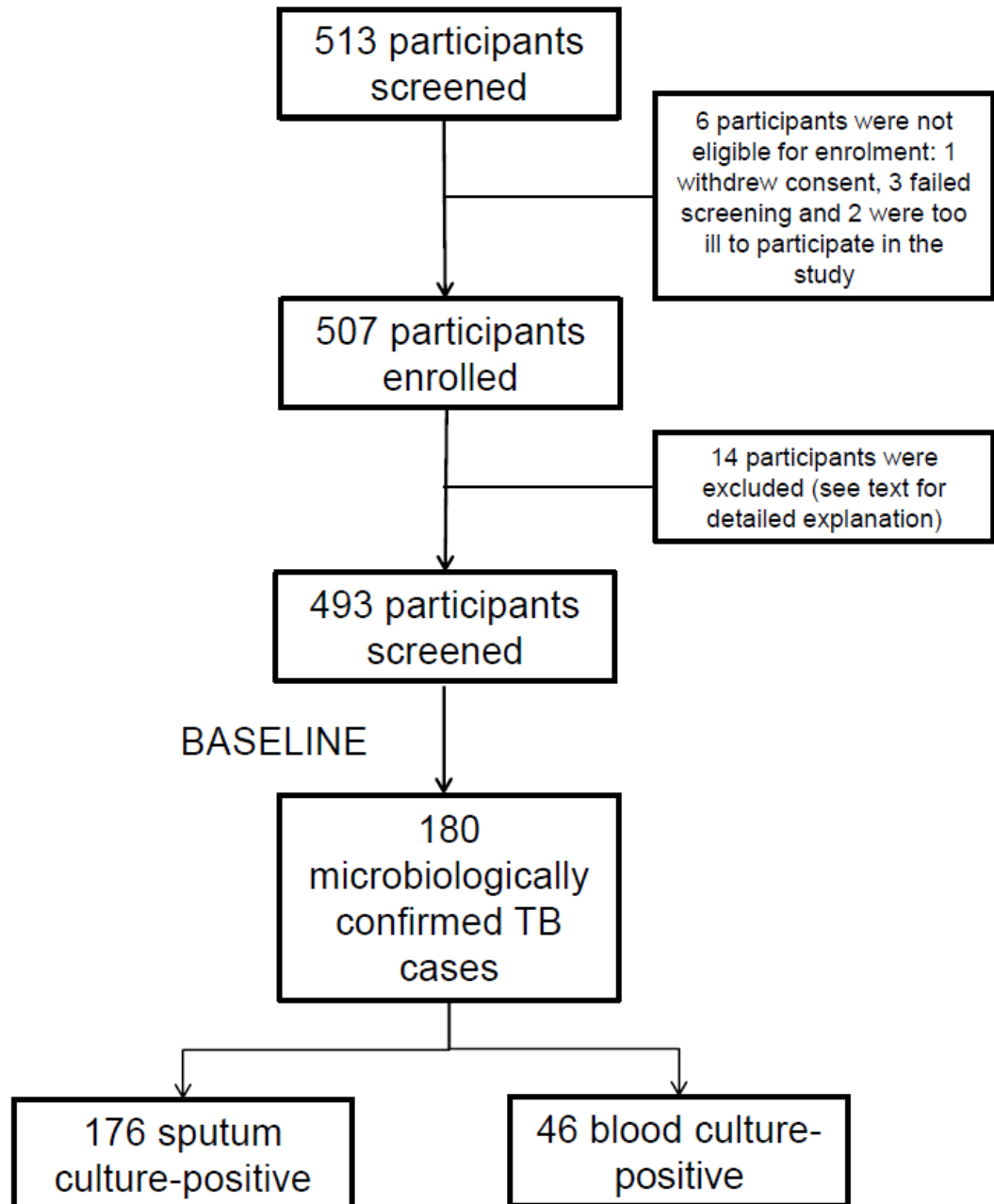


Figure 7. Schematic depicting study flow with results of microbiological culture.

The baseline characteristics of the participants are shown in Table 1. Two hundred of the 493 (40.6%) of participants were hospitalised patients at GF Jooste Hospital. The two groups (hospitalised versus outpatients) differed in median weight and median CD4 T-cell count. Using a Z-score two population proportion calculation, significantly more hospitalised participants had CD4 T-cell counts <100 cells/mm³ ($p<0.001$) [Table 2]. More outpatients had CD4 T-cell counts >200 cells/mm³ ($p<0.001$) and reported symptoms of cough ($p=0.002$), night sweats ($p<0.001$) and loss of appetite ($p<0.001$) compared to hospitalised patients. There was a statistically significant greater number of hospitalised patients on antiretroviral drugs compared to outpatients ($p<0.001$).

Table 1. Comparison of clinical characteristics between hospitalised patients and outpatients, expressed as medians [IQR] or frequencies (%).

Characteristic	Entire cohort (n=493)	Hospitalised patients (n=200)	Outpatients (n=293)	p-value
Median age in years [IQR]	35 [28.0-41.0]	ND	ND	UC
Male Gender	181 (36.7%)	70 (35.0%)	111(37.9%)	0.52
Median weight in kilograms [IQR]#	60.7 [52.7-71.2]	56.0 [48.6-65.6]	64.0 [57.0-75.0]	<0.001
Median CD4 T-cell count (cells/mm3)[IQR]*	215 [87.0-389.0]	120.5 [46.5-277.0]	270 [155.0-449.0]	<0.001
Antiretroviral therapy#				
Ever prescribed	146 (29.7%)	86 (43.0%)	60 (20.5%)	<0.001
Currently on	139 (28.3%)	79 (39.5%)	60(20.5%)	<0.001
Co-trimoxazole therapy§				
Ever prescribed	147 (30.0%)	66 (33.3%)	81(27.7%)	0.18
Currently on	134 (27.3%)^	53 (26.8%)	81(27.7%)	0.81
Symptoms#				
Cough	486 (98.8%)	194 (97.0%)	292 (100%)	0.002
Fever within past 4 weeks	363 (73.8%)	150 (75.0%)	213 (72.9%)	0.61
Night sweats within past 4 weeks	427 (86.8%)	144 (72.0%)	283 (96.9%)	<0.001
Fatigue	444 (90.2%)	183 (91.5%)	261 (89.4%)	0.44
Poor appetite	432 (87.8%)	163 (81.5%)	269 (92.1%)	<0.001
Weight loss within past 4 weeks	472 (95.9%)	190 (95.0%)	282 (96.6%)	0.38

Key:

IQR: interquartile range

ND: data not available

UC: unable to compute

*Baseline CD4 count was missing for two outpatients

#Baseline clinical symptom and antiretroviral treatment information was missing for one outpatient

§ Co-trimoxazole treatment information was missing on three participants (two hospitalised, one outpatient)

Table 2. Stratified comparison of weight and CD4 T-cell count between hospitalised patients and outpatients, expressed as frequencies (%).

Characteristic	Subcategory	Entire cohort (n=493)	Hospitalised patients (n=200)	Outpatients (n=293)	p-value
Weight in kilograms#	<50	86 (17.5)	62 (31.0)	24 (8.2)	<0.001
	50-70	273 (55.5)	107 (53.5)	166 (56.8)	0.47
	71-100	122 (24.8)	26 (13.0)	96 (32.9)	< 0.001
	>100	11 (2.2)	5 (2.5)	6 (2.1)	0.74
CD4 T-cell count (cells/mm ³)*	≤50	83 (16.9)	55 (27.5)	28 (9.6)	<0.001
	51-99	51 (10.4)	33 (16.5)	18(6.2)	<0.001
	100-199	97 (19.8)	46 (23.0)	51(17.5)	0.13
	≥200	260 (53.0)	66 (33.0)	194(66.7)	<0.001

Key:

#Baseline clinical, symptom and treatment information was missing on one outpatient

*Baseline CD4 count was missing for two outpatients

At baseline, one hundred and eighty (36.5%) of 493 participants were confirmed mycobacterial culture-positive (sputum MGIT and/or LJ and/or blood culture) [Figure 8]. LJ culture only contributed one (0.6 %) additional sputum culture-positive participant. Seventy-four (15%) of the 493 participants were Ziehl-Neelsen smear-positive whilst 98 (19.9%) participants were auramine smear-positive. One hundred and two participants (20.7%) had at least one positive smear microscopy (ZN and/or auramine) result. Forty-six (9.3%) of 492 participants had a positive blood culture with confirmed *M. tuberculosis* complex. Blood culture yielded four additional culture-positive participants whom were sputum culture-negative. Four hundred and eighty-seven (98.8%) of 493 participants had two sputum specimens cultured. Of the remaining six (1.2%) participants who had a single sputum specimen cultured, five were unable to produce a second specimen and for one participant, the specimen was lost in transit to the laboratory. Four of these six participants were confirmed sputum culture-positive on their single specimen.

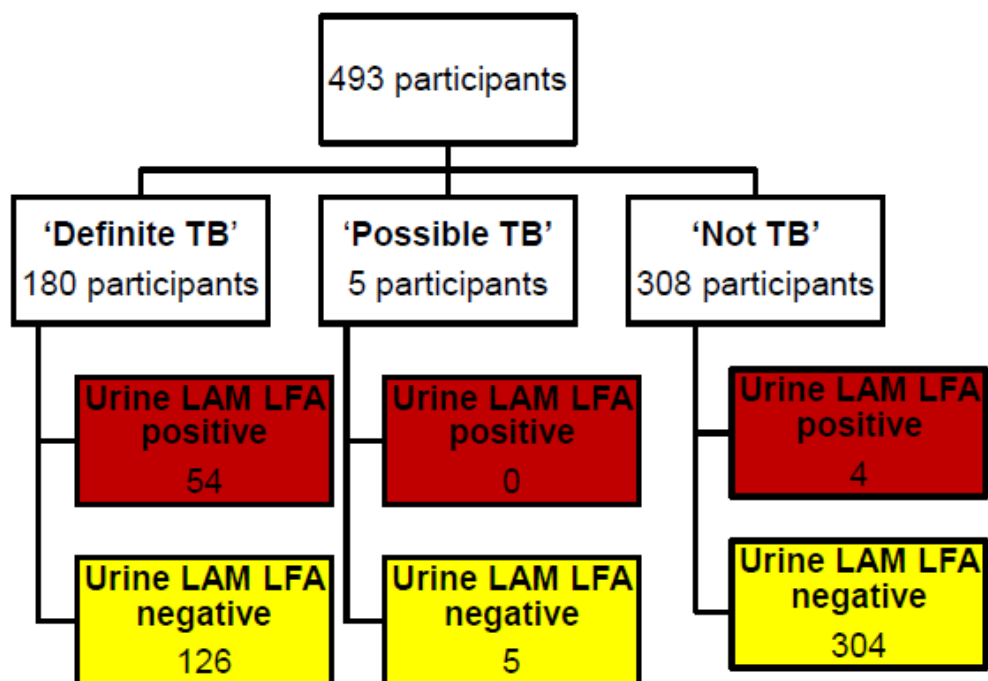


Figure 8. Breakdown of urine LAM test results by case definition.

4.2 Sensitivity and specificity of the different tests

4.2.1 Determine™ urine LAM LFA

A valid urine LAM LFA result was obtained on first attempt for all 493 (100%) participants. The sensitivity and specificity of the urine LAM LFA using 'Definite TB' as the reference standard is shown in Table 3. The sensitivity and specificity of the urine LAM LFA as read by reader one (band intensity $\geq$ Grade 2) was 30% (95% CI [23.4, 37.3]) and 98.7% (95% CI [96.7, 99.7]), respectively. This was similar to the sensitivity and specificity when the readings were performed by reader one at late reading, whilst the readings by reader 2 only marginally improved sensitivity compared to reader 1 at the expense of a loss of specificity (Table 3).

Table 3. Sensitivity, specificity, PPV and NPV using 'Definite TB' (positive mycobacterial culture of sputum and blood at baseline) as a reference standard.

Readers	Urine LAM LFA results	Sensitivity (%), [95% CI]	Specificity (%), [95% CI]	PPV (%), [95% CI]	NPV (%), [95% CI]
Reader One	<i>Band intensity ≥ Grade 2</i>	30.0% [23.4, 37.3]	98.7% [96.7, 99.7]	93.1% [83.3, 99.0]	70.7% [66.2, 75.0]
	<i>Any band positive</i>	57.8% [50.2, 65.1]	76.6% [71.5, 81.2]	59.1%, [51.4, 66.4]	75.6% [70.5, 80.3]
	<i>Late read- Band intensity ≥ Grade 2</i>	29.4% [22.9, 36.7]	98.7 % [96.7, 99.7]	93.0% [83.0, 98.1]	70.5% [66.0, 74.8]
Reader Two	<i>Band intensity ≥ Grade 2</i>	33.9% [27.0, 41.3]	97.1 % [94.5, 98.7]	87.1% [77.0, 94.0]	71.5% [66.9, 75.8]

Key: PPV-predictive value of a positive test; NPV-predictive value of a negative test.

The sensitivity and specificity of the assay when used in hospitalised patients versus outpatients is shown in Table 4. The sensitivity was significantly better in hospitalised patients (44.2 %; 95% CI [34.0, 54.8]) compared to outpatients (14.1%; 95% CI [7.5, 23.4]; $p<0.05$).

Table 4. Sensitivity, specificity, PPV and NPV using ‘Definite TB’ (positive mycobacterial culture of sputum and/or blood at baseline) as a reference standard stratified according to hospitalisation status.

	Hospitalised	Outpatients
<i>Sensitivity [95% CI]</i>	44.2% [34.0, 54.8]	14.1% [7.5, 23.4]
<i>Specificity [95% CI]</i>	98.0% [93.1, 99.8]	99.0% [96.5, 99.9]
<i>PPV [95% CI]</i>	95.5% [84.5, 99.4]	85.7% [57.2, 98.2]
<i>NPV [95% CI]</i>	65.4% [57.3, 72.9]	73.7% [68.0, 78.7]

Amongst the ZN smear-negative participants (417/488), the sensitivity of the urine LAM LFA was 22.0% (95% CI [14.7, 31.0]) and specificity was 98.7% (95% CI [96.7, 99.7]). Thirty of 58 (51.7%) participants with a positive blood culture were also urine LAM LFA positive. Of note, of the five participants with non-tuberculous mycobacteria detected on sputum, only one participant had a positive urine LAM result (Grade 2 band).

Sixty-nine patients had repeat urine LAM LFAs performed at two month follow-up. Forty-one (59.4%) of these LAM LFAs remained positive. None of these participants had a positive TB culture at two months follow-up implying these were likely false-positive LAM LFA results.

As per the readings of the urine LAM LFA performed by reader one, the distribution of band intensity results were: no band (Grade 0), 312 (63.9%); Grade 1 (+/-), 118 (24.2%); Grade 2 (1+), 17 (3.5%); Grade 3 (2+), 13 (2.7%); Grade 4 (3+); 21 (4.3%); and Grade 5 (4+ or greater), 7 (1.4%). The results for reader 1, reader 2 and late read are graphically depicted in Figure 9. The inter-reader agreement between reader 1 and reader 2 as to presence or absence of a band of intensity $\geq$ Grade 2 was very good ($\kappa=0.9$; 95%CI [0.8, 0.9]).

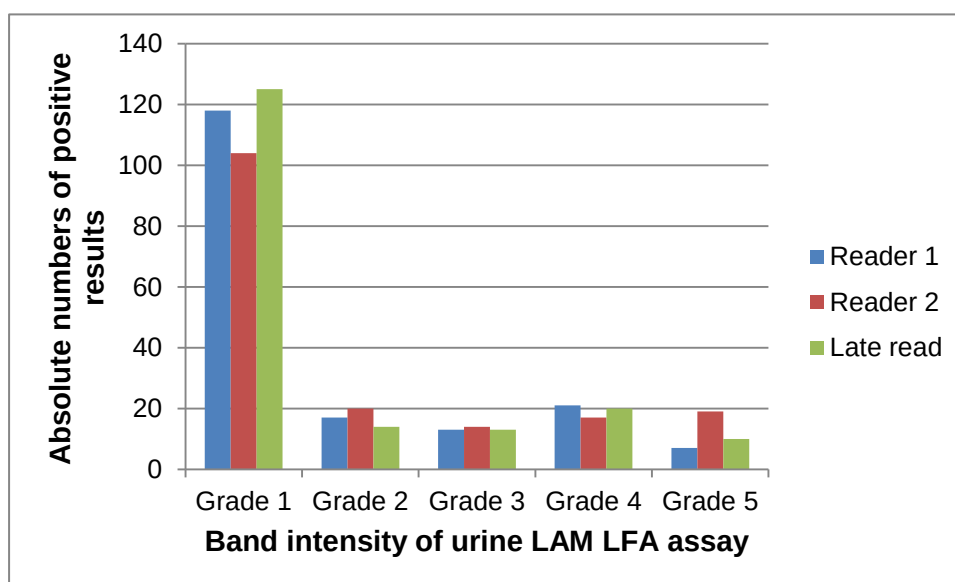


Figure 9. Positive urine Determine™ LAM LFA results by band intensity.

Receiver-operating curve analysis (ROC) revealed that a test band intensity positive threshold of Grade 2 maximised sensitivity and specificity of the assay (Figure 10).

Determine Urine LAM LFA Receiver Operator Characteristic Curve

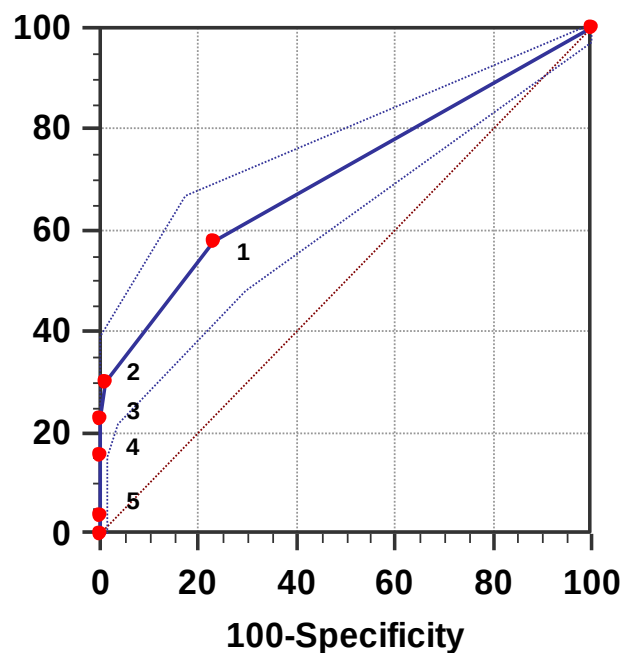


Figure 10. Receiver-operator characteristic curve of band intensity for the Determine™ TB LAM LFA assay.

Key: The integers adjacent to points correspond to band intensity of $\geq$ grade 1 (1), $\geq$ grade 2 (2), $\geq$ grade 3 (3), $\geq$ grade 4 (4) and $\geq$ grade 5 (5), respectively. The area under the curve (AUC) $\pm$ standard error was 0.71 ± 0.02 ($p < 0.001$). The blue dotted lines indicate 95% confidence intervals [0.66, 0.75]. The brown dotted line is the reference line.

4.2.2 Clearview™ urine LAM ELISA

The sensitivity and specificity of the Clearview™ urine LAM ELISA was 38.9% (95% CI [31.7, 46.4]) and 99.7% (95% CI [98.2, 100.0]) respectively when using 'Definite TB' as a reference standard (Table 5). The sensitivity and specificity of the urine LAM LFA compared to the Clearview™ LAM ELISA as the reference standard is also shown in Table 5. All 19 urine ELISA-positive, urine LAM LFA-negative participants were confirmed TB culture-

positive. There were six participants who were urine ELISA-negative, urine LAM LFA-positive of which three were culture-confirmed TB-positive. Fifty eight (11.9%) participants' urine LAM ELISA tests were invalid and needed to be repeated. Of these, only one (0.2%) participant had an invalid result on repeat testing.

Table 5. The sensitivity and specificity of the different urine LAM tests using 'Definite TB' as well as the Clearview™ ELISA as the reference standards, respectively.

Diagnostic tests	Definite TB as reference standard		Clearview™ LAM ELISA as reference standard	
	Sensitivity	Specificity	Sensitivity	Specificity
<i>ClearView™ LAM ELISA</i>	38.9% [31.7, 46.4]	99.7% [98.2, 100.0]	NC	NC
<i>Determine™ urine LAM LFA</i>	30.0% [23.4, 37.3]	98.7% [96.7, 99.7]	73.2% [61.4, 83.1]	98.6% [96.9, 99.5]

Key: NC-not calculated

4.2.3 Incremental yield of urine LAM combined with microscopy and X-ray

TB LAM LFA sensitivity was similar to direct ZN smear microscopy (30% versus 39.4%, $p=0.06$) [Table 6], but lower than that of auramine (53.33%, 95% CI [45.8, 60.8]). The combined sensitivity of the TB LAM LFA with direct ZN smear microscopy was higher than the sensitivity of either test alone (52.8%, 95% CI [45.2, 60.3]). The sensitivity of combining the urine LAM with ZN and chest radiography was 82.3% (95% CI [75.6, 87.8]); however, specificity was 59.5% (95% CI [53.2, 65.5]). Concentrated auramine test results were not included in the analyses mentioned previously as the intent of the study was to investigate the use of the LAM LFA at peripheral microscopy centres in

low- and middle- income settings where concentrated auramine is not routinely available.⁽⁶⁸⁾

Table 6. Sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) for the various TB diagnostics using 'Definite TB' as the reference standard.

Tests	Sensitivity [95% CI]	Specificity [95% CI]	PPV [95% CI]	NPV [95% CI]
Microscopy				
Direct ZN staining sputum	39.4% [32.3, 47.0]	100.0% [98.8, 100.0]	100.0% [94.9, 100.0]	73.9% [69.4, 78.0]
Concentrated auramine staining sputum	53.3% [45.8, 60.8]	100.0% [98.8, 100.0]	100.0% [96.2, 100.0]	78.6% [74.2, 82.5]
CXR (<i>n</i> =423)	78.1% [70.9, 84.1]	59.9% [53.6, 65.9]	55.2% [48.5, 61.7]	81.2% [74.9, 86.4]
Clearview™ LAM ELISA urine (<i>n</i> =487) ^ψ	38.9% [31.7, 46.4]	99.7% [98.2, 100.0]	98.6% [92.4, 100.0]	73.6% [69.0, 77.7]
Combinations of tests				
LAM LFA urine + ZN staining sputum	52.8% [45.2, 60.3]	98.7% [96.7, 99.7]	96.0% [90.0, 98.9]	78.2% [73.7, 82.2]
LAM LFA urine + CXR	81.7% [74.9, 87.3]	59.5% [53.2, 65.5]	56.1% [49.5, 62.5]	83.7% [77.6, 88.7]
LAM LFA urine+ ZN staining sputum + CXR	82.3% [75.6, 87.8]	59.5% [53.2, 65.5]	56.3% [49.7, 62.6]	84.2% [78.0, 89.1]

Key: ^ψ One participant's test remained invalid on repeat testing and was thus excluded from analysis.

ZN Ziehl-Neelsen stain

CXR Chest radiography

4.3 Characteristics associated with a positive urine LAM LFA test

Table 7 shows univariate associations which are unadjusted for confounders.

Table 7. Univariate analysis of the clinical characteristics of participants with positive or negative urine LAM LFA test results, expressed as frequencies (%).

Clinical characteristic	Overall participants, <i>n</i> =483*	Participants with positive LAM LFA result, <i>n</i> =58	Participant with negative LAM LFA result, <i>n</i> =425	<i>p</i> -value
Hospitalised, <i>n</i> (%)	195 (40.4)	44 (75.9)	151 (35.5)	<0.001
Cough, <i>n</i> (%)	477 (98.8)	55 (94.8)	422 (99.3)	0.03
Night sweats, <i>n</i> (%)	420 (87.0)	48 (82.8)	372 (87.5)	0.31
Fatigue, <i>n</i> (%)	436 (90.3)	57 (98.3)	379 (89.2)	0.03
Poor appetite, <i>n</i> (%)	425 (88.0)	50 (86.2)	375 (88.2)	0.66
Fever, <i>n</i> (%)	356 (73.7)	42 (72.4)	314 (73.9)	0.81
Weight loss, <i>n</i> (%)	464 (96.1)	58 (100.0)	406 (95.5)	0.15
Antiretrovirals ever prescribed, <i>n</i> (%)	140 (29.0)	13 (22.4)	127 (29.9)	0.24
Antiretrovirals currently, <i>n</i> (%)	133 (27.5)	12 (20.7)	121 (28.5)	0.21
Cotrimoxazole ever prescribed, <i>n</i> (%)	144 (29.8)	12 (20.7)	132 (31.1)	0.11
Cotrimoxazole currently, <i>n</i> (%)	131(27.1)	11 (19.0)	120 (28.2)	0.14
Karnofsky score <80, <i>n</i> (%)	104 (21.5)	28 (48.3)	76 (17.9)	<0.001
CD4 count (cells/mm ³)				<0.001
≤ 50, <i>n</i> (%)	82 (17.0)	31 (53.4)	51 (12.0)	
51-99, <i>n</i> (%)	50 (10.4)	15 (25.9)	35 (8.2)	
100-199, <i>n</i> (%)	96 (19.9)	6 (10.3)	90 (21.2)	
≥ 200, <i>n</i> (%)	255 (52.8)	6 (10.3)	249 (58.6)	
Weight in kilograms, <i>n</i> (%)				0.002
<50	86 (17.8)	18 (31.0)	68 (16.0)	
50-70	266 (55.1)	34 (58.6)	232 (54.6)	
71-100	121 (25.1)	5 (8.6)	116 (27.3)	
>100	10 (2.1)	1 (1.7)	9 (2.1)	

Key: * *n*=483 participants (five participants were missing baseline symptom data and five participants who were classified as 'Possible TB' were excluded).

Using logistic regression analysis (Table 8), patients with CD4 T-cell counts <50 cells/mm³ had a 15-fold higher odds of testing positive on the LAM LFA when compared with the reference group (CD4 T-cell count ≥200 cells/mm³). Patients with CD4 T-cell counts of 51-99 cells/mm³ had 11-fold higher odds of testing positive on the LAM LFA when compared with the reference group. There was no difference in the odds of a positive LAM LFA between patients with CD4 T-cell counts of 100-199 cells/mm³ and the reference group (CD4≥200 cells/mm³). There was no difference in the odds of a positive LAM test between the reference group (weight>100kg) and all other weight categories.

Table 8. Multivariate analysis of clinical characteristics associated with a positive urine LAM test result.*

Clinical characteristic	Odds Ratio (95% CI)	p-value
<i>Cough</i>	0.14 (0.02, 1.17)	0.07
<i>Fatigue</i>	6.46 (0.64, 65.57)	0.12
<i>Karnofsky score <80</i>	1.22 (0.56, 2.64)	0.62
<i>Hospitalisation</i>	2.24 (0.99, 5.05)	0.05
<i>CD4 count (cells/mm³)</i>		
≤50	15.06 (5.67, 40.01)	<0.001
51-99	11.31 (3.92, 32.58)	<0.001
100-199	1.84 (0.55, 6.15)	0.32
≥200	Reference	
<i>Weight (kilograms)</i>		
≤ 50	0.68 (0.05, 8.92)	0.77
51-70	0.66 (0.05, 8.49)	0.75
71-100	0.33 (0.02, 4.90)	0.421
>100	Reference	

*Weight loss, night sweats, poor appetite, and fever did not meet the criteria of p<0.1 from the univariate analysis for inclusion in the multivariate analysis (logistic regression).

The characteristics of those patients with positive urine LAM LFAs, stratified by hospitalisation status, are depicted in Table 9. Significantly more LAM LFA-positive hospitalised patients had Karnofsky scores ≤ 80 .

Table 9. Patient characteristics of those with a positive urine LAM LFA test stratified by hospitalisation status, expressed as frequencies (%).

			LAM LFA positive		
	Total population with positive characteristic (n=483)	Total LAM LFA positive (n=58)	Hospitalised (n=44)	Outpatient (n=14)	p-value
Symptoms					
Cough	477 (98.8)	55 (94.8)	41 (92.2)	14 (100.0)	1.00
Night sweats	420 (87.0)	48 (82.8)	34 (77.3)	14 (100.0)	0.09
Fatigue	436 (90.3)	57 (98.3)	44 (100.0)	13 (92.9)	0.24
Poor appetite	425 (88.0)	50 (86.2)	36 (81.8)	14 (100.0)	0.18
Fever	356 (73.7)	42 (72.4)	33 (75.0)	9 (64.3)	0.50
Weight loss	464 (96.1)	58 (100.0)	44 (100.0)	14 (100.0)	NC
Antiretrovirals					
Ever prescribed	140 (29.0)	13 (22.4)	11 (25.0)	2 (14.3)	0.49
Currently on	133 (27.5)	12 (20.7)	10 (22.7)	2 (14.3)	0.71
Cotrimoxazole					
Ever prescribed	144 (29.8)	12 (20.7)	10 (22.7)	2 (14.3)	0.71
Currently on	131 (27.1)	11 (19.0)	9 (20.5)	2 (14.3)	1.00
Karnofsky score <80	104 (21.5)	28 (48.3)	26 (59.1)	2(14.3)	0.003
Weight (kilograms)					
<50	86 (17.8)	18 (31.0)	16 (36.4)	2 (14.3)	
50-70	266 (55.1)	34 (58.6)	24 (54.5)	10 (71.4)	
71-100	121 (25.1)	5 (8.6)	3 (6.8)	2 (14.3)	
>100	10 (2.1)	1 (1.7)	1 (2.3)	0 (0.0)	
CD4 T-cell count (cells/mm ³)					
≤50	82 (17.0)	31 (53.4)	26	5 (35.7)	
51-99	50 (10.4)	15 (25.9)	11	4 (28.6)	
100-199	96 (19.9)	6 (10.3)	5	1 (7.1)	
≥200	255 (52.8)	6 (10.3)	2	4 (28.6)	

Key: NC unable to compute

4.4 Urine LAM test results stratified according to degree of immunosuppression as indicated by CD4 T-cell count

The sensitivity and specificity of the Determine™ urine LAM LFA assay according to CD4 T-cell count is depicted in Figure 11. The sensitivity was highest in the patient group with CD4 T-cell counts ≤ 50 cells/mm³.

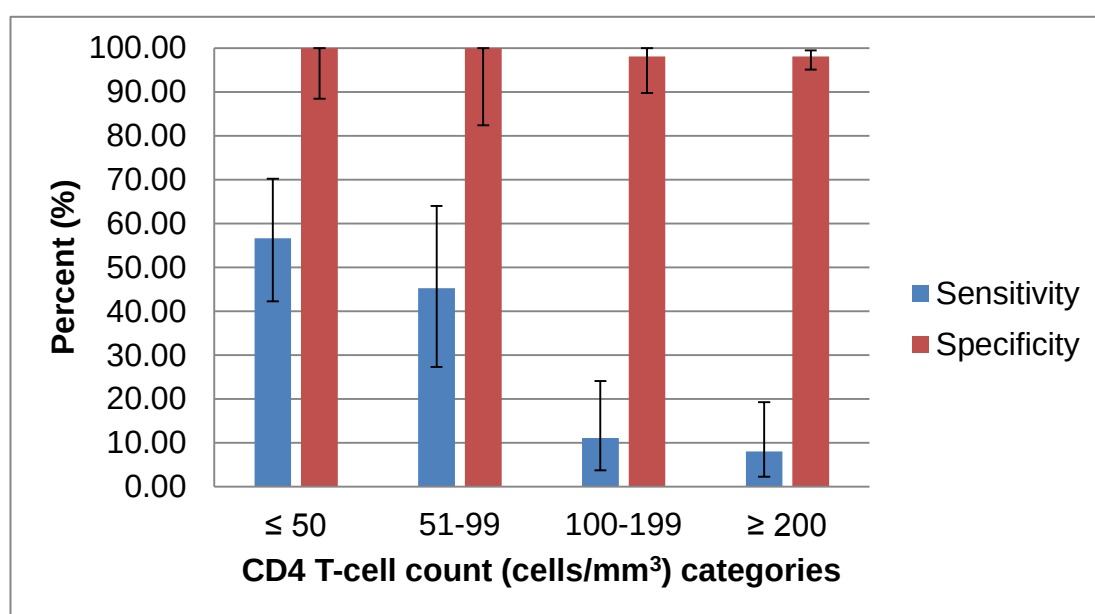


Figure 11. Sensitivity and specificity of urine LAM according to CD4 T-cell enumeration.

Key: Error bars indicate 95% confidence intervals.

4.5 Antituberculosis therapy

According to their past medical history, one hundred and five (21.3%) of 492 participants had been prescribed TB treatment previously. Twenty-eight (5.7%) of the 492 participants were currently on multidrug TB treatment (< 2

days therapy). Of these participants, 13 (46.4%) were urine LAM LFA positive. Of these 13 participants, 12 (92.3%) were confirmed culture-positive. Of the remaining 15 participants on TB treatment and with negative urine LAM LFAs, eight were confirmed culture-positive. Six of these eight culture-positive participants had urine LAM LFAs with Grade 1 (+/-) band intensity and would have been detected if a lower positive cut-point was used for interpretation of the LAM LFA result.

4.6 Proteinuria

Urine dipstick analysis revealed 275/488 (56.4%) participants had some evidence of proteinuria (Figure 12). Seventeen of 68 (25.0%) participants with proteinuria ≥ 300 mg/dL had a positive urine LAM LFA whereas only 6.1% (13/213) of participants with no proteinuria had a positive urine LAM LFA ($p < 0.05$).

Although TB culture of urine specimens was not performed in this study, record review revealed that 18 participants had a urine TB culture performed by their treating physician. Of these, only one participant was culture-positive (but LAM LFA-negative), whilst 15 participants were culture-negative. The remaining two participants had contaminated cultures. Three of the 15 (20.0%) participants with a negative urine TB culture result had positive urine LAM LFAs [one participant with a Grade 5 result; one participant with a Grade 3 result; and one participant with a Grade 2 result]. Four participants with a negative urine TB culture result had a Grade 1 urine LAM LFA result. Only one of these four participants had a positive sputum culture.

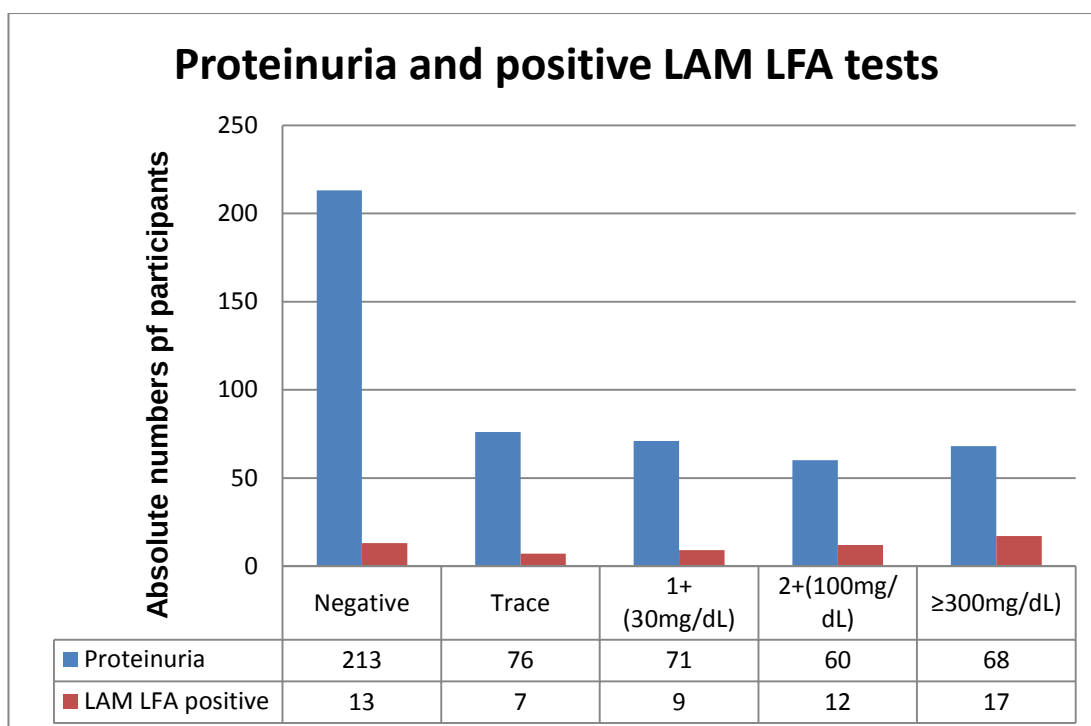


Figure 12. Correlation between degree of proteinuria and urine LAM LFA positive results.

4.7 Chest radiography

Twenty seven (22.7%) of 119 participants with a CXR highly suggestive of TB had a positive urine LAM LFA at baseline (Figure 13). Of 113 participants with abnormal CXRs that could be compatible with TB, 16 (14.2%) has a positive urine LAM LFA.

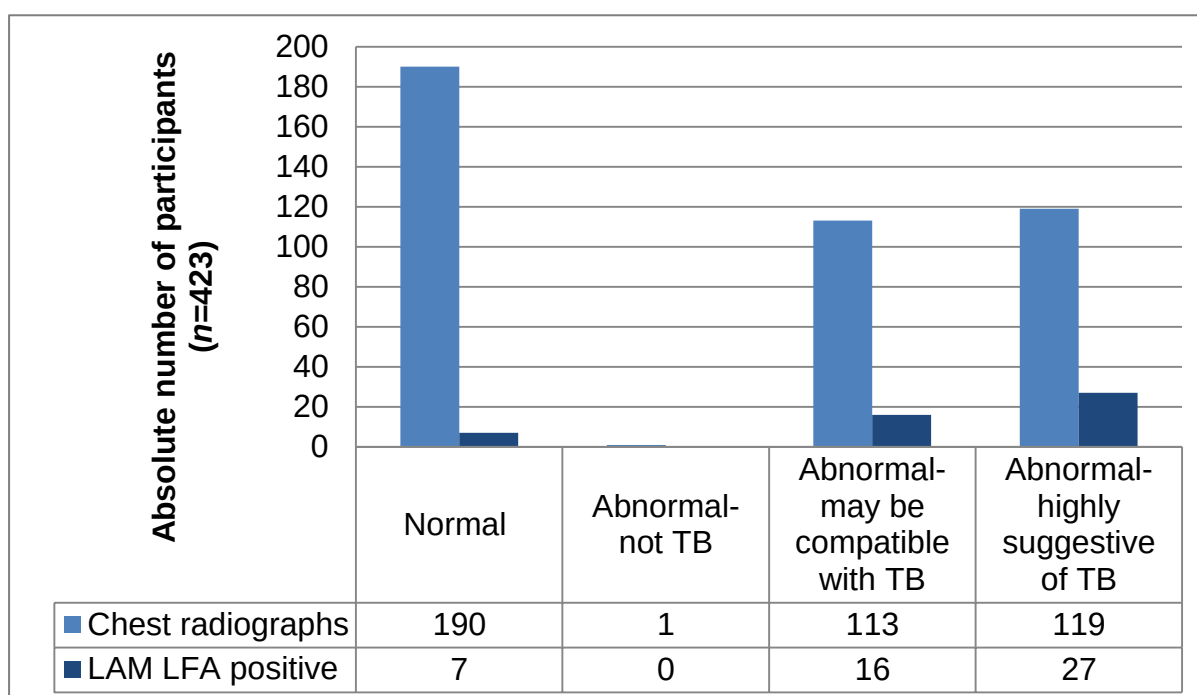


Figure 13. Correlation between chest radiographs and urine LAM LFAs (both performed at baseline).

5. DISCUSSION

Our study found that the urine LAM LFA detected over half of TB-infected hospitalised participants with advanced immunosuppression (CD4 T-cell count <100 cells/mm³). This is particularly valuable as this is the group that exhibits the highest morbidity and mortality. The test specificity exceeded 95%, the inter-reader agreement was high and a valid result was obtained on first attempt on all participants, proving that it has a role as a useful POC test. The combination of urine LAM LFA and direct smear microscopy had higher sensitivity than did either test used alone.

Our study differs from previously published studies in that it has fulfilled most of the recommendations for reporting of clinical studies for the Determine™ TB LAM LFA for the diagnosis of TB as recommended by Lawn *et al.*⁽⁶¹⁾ This was a prospective study that included known HIV-infected hospitalised patients as well as HIV-infected outpatients with low CD4 T-cell counts. In addition, the patient population was characterised using parameters such as symptom profile, CD4 T-cell count, and Karnofsky score. We cultured multiple clinical samples including sampling from sites of disease for extrapulmonary TB (i.e. blood culture). The microbiological testing was performed in a quality-assured laboratory using liquid culture methodology. Sputum samples were carefully obtained, including sputum induction using nebulised hypertonic saline in patients unable to expectorate spontaneously and more than one sputum sample was tested in light of the significant incremental yield. The species of the cultured isolates of mycobacteria were confirmed by molecular methods. Additionally, we performed the Determine™ urine LAM LFA on freshly voided urine within 24 hours of specimen receipt as opposed to testing on frozen specimens. The spectrum of participants included in the study is representative of the patients who will receive the test in practice (no spectrum composition bias), the reference standard is independent of the index test (incorporation bias), and tests were interpreted without knowledge of other test results (reference standard bias).

5.1 Sensitivity and specificity of urine LAM LFA

Our study found that the Determine® urine LAM LFA assay had an overall sensitivity of 30% (95% CI [23.4, 37.3]) and a specificity of 98.7% (95% CI [96.7, 99.7]) [Table 3]. In the Ugandan arm (not reported here), we found that the overall sensitivity was higher at 45.6% (95% CI [38.2, 53.1]) with a specificity of 95% (95% CI [91.8%, 97.3]).⁽⁶⁹⁾ This difference in sensitivity

between the two study sites is likely due to differences in disease severity at primary presentation. In support of this hypothesis, the sensitivity across both sites using any visualised band as a positive test for hospitalised participants with CD4 T-cell counts ≤ 100 cells/mm³ was similar [71/109 (65.1%; 95% CI [55.4, 74.0]) in Uganda and 65/84 (77.4%; 95% CI [67.0, 85.8]) in Cape Town ($p=0.06$)].⁽⁶⁹⁾ It is possible though that other factors such as mycobacterial strain type, test interpretation by technicians, or batch variation in manufacture of the LAM LFA may have also played a role in the difference.⁽⁶⁹⁾

The results of other urine LAM LFA studies from Sub-Saharan Africa are shown in Table 10. Some of these authors reported similar sensitivity and specificity to that found on our study.^(55, 70, 71) A recent Cochrane review⁽⁷²⁾ evaluated five studies^(56, 59, 69, 73, 74) for diagnosis of active TB in HIV-infected participants. Using a grade 2 cut-off, the meta-analysis showed median pooled sensitivity and specificity were 45% (95% Credible Interval (CrI) [29, 63]) and 92% (95% CrI [80, 97]), respectively (2,313 participants; 35% with TB).⁽⁷²⁾

Similar to our study, Peter *et al.*⁽⁵⁹⁾ found in their multicentre study that LAM specificity was significantly higher at the South African sites (99% in Cape Town, and 97% in Durban) compared to the Zambian (87.1% in Lusaka) and Tanzanian (89.7% in Mbeya) study sites, both in their primary and secondary analyses. The authors attributed lower specificity due to “unavoidable misclassification bias associated with a single sputum culture to correctly classify TB” in their cohort of HIV-infected patients with advanced immunosuppression (i.e. misclassification of TB patients as non-TB).⁽⁵⁹⁾ Drain *et al.*⁽⁷⁰⁾[Table 10-*c*], in contrast to our study, collected only a single sputum for AFB microscopy and mycobacterial culture as well as regarded a band of any intensity as a positive result, possibly accounting for the lower specificity

reported in their study. In addition, the authors performed the urine LAM on frozen urine in 14% (48/342) of participants due to interrupted supply of the test kit which may have affected test performance.⁽⁷⁰⁾

Table 10. Summary of recent studies evaluating the performance of the Determine™ urine LAM LFA in sub-Saharan Africa.

Study	Country	LAM assay	Study design	Total number of participants	Participant characteristics	HIV positive included	Reference test/no. of specimens cultured (per participant)	Fresh/frozen urine	Urine LAM LFA cutoff used	Sensitivity (95% CI)	Specificity (95% CI)
Our study	South Africa	Alere Determine™ LAM LFA	Prospective cross-sectional accuracy	493	Outpatients and hospitalised	Yes (n=493)	Liquid culture+solid culture (LJ)+blood culture (2 sputum; 1 blood culture)	Fresh	Grade 2	30.0% (23.4, 37.3)	98.7% (96.7, 99.7)
Lawn <i>et al.</i> #	South Africa	Alere Determine™ LAM LFA	Prospective cross-sectional descriptive	516	Outpatients	Yes (n=516) Included HIV-positive and participants who refused testing	Liquid culture and Xpert MTB/RIF assay (2 sputa)	Frozen	Not specified	28.2% (19.0, 39.0)	98.6% (97.0, 99.5)
Peter <i>et al.</i> *	South Africa; Zambia and Tanzania	Alere Determine™ LAM LFA	Prospective cross-sectional accuracy	1095	Outpatients	Yes (n=564)	Liquid culture (1 sputum)	Frozen	Grade 2	22.7 % (16.6, 28.7) [HIV infected group]	93.0% (90.5, 95.6) [HIV infected group]
Drain <i>et al.</i> †	South Africa	Alere Determine™ LAM LFA	Prospective cross-sectional	342	Outpatients	Yes (n=342)	Liquid culture+solid culture (Middlebrook 7H11 solid agar medium) [1 sputum]	Fresh and frozen	Any band considered positive	28.3% (17.5, 41.4)	90.1% (86.0, 93.3)
Drain <i>et al.</i> ‡	South Africa	Alere Determine™ LAM LFA	Prospective cross-sectional	320	Outpatients	Yes (n=320)	Liquid culture+solid culture (Middlebrook 7H11 solid agar medium) [1 sputum]	Fresh	Grade 2	28.0% (16.0, 42.0)	94.0% (90.0, 97.0)
Peter <i>et al.</i> ¶	South Africa	Alere Determine™ LAM LFA	Prospective cross-sectional	423	Hospitalised	Yes (n=242)	Not standardised	Frozen	Grade 2	50.0% (41.0, 59.0) using microbiological reference	75.0% (67.0, 82.0)
Peter <i>et al.</i> ψ	South Africa	Alere Determine™ LAM LFA	Prospective cross-sectional accuracy	242	Hospitalised	Yes (n=242)	Liquid culture (2 sputa)+ culture of extrapulmonary specimens if indicated	Frozen	Grade 2	48.0% (39.0-57.0)	85.0% (77.0-94.0)

Lawn SD, Kerkhoff AD, Vogt M, Wood R. Diagnostic accuracy of a low-cost, urine antigen, point-of-care screening assay for HIV-associated pulmonary tuberculosis before antiretroviral therapy: a descriptive study. *Lancet Infect Dis.* 2012;12(3):201-209. Epub 2011 Oct 17. doi: 10.1016/S1473-3099(11)70251-1

* Peter J, Theron G, Chanda D, Clowes P, Rachow A, Lesosky M, et al. Test characteristics and potential impact of the urine LAM lateral flow assay in HIV-infected outpatients under investigation for TB and able to self-expectorate sputum for diagnostic testing. *BMC Infect Dis.* 2015;15:262. doi: 10.1186/s12879-015-0967-z

† Drain PK, Losina E, Coleman SM, Giddy J, Ross D, Katz JN, et al. Diagnostic accuracy of a point-of-care urine test for tuberculosis screening among newly-diagnosed HIV-infected adults: a prospective, clinic-based study. *BMC Infect Dis.* 2014;14:110. doi: 10.1186/1471-2334-14-110

‡ Drain PK, Losina E, Coleman SM, Giddy J, Ross D, Katz JN, et al. Value of urine liparabinomannan grade and second test for optimizing clinic-based screening for HIV-associated pulmonary tuberculosis. *J Acquir Immune Defic Syndr.* 2015;68(3):274-280. doi: 10.1097/QAI.0000000000000436

¶ Peter JG, Theron G, van Zyl-Smit R, Haripersad A, Mottay L, Kraus S, et al. Diagnostic accuracy of a urine liparabinomannan strip-test for TB detection in HIV-infected hospitalised patients. *Eur Respir J.* 2012;40(5):1211-1220. Epub 2012 Feb 23. doi: 10.1183/09031936.00201711

ψ Peter JG, Theron G, Muchinga TE, Govender U, Dheda K. The diagnostic accuracy of urine-based Xpert MTB/RIF in HIV-infected hospitalized patients who are smear-negative or sputum scarce. *PLoS One.* 2012; 7(7): e39966. Epub 2012 Jul 09. doi: 10.1371/journal.pone.0039966

Additional factors that may contribute to varying specificity across study sites are environmental, bacterial or fungal contaminants of urine.⁽⁷⁵⁾ Kroidl *et al.*⁽⁷⁶⁾ found false positives arising from contamination of the LAM ELISA with dust, soil and stool in Tanzania, and Dheda *et al.*⁽⁴⁸⁾ determined that anti-LAM polyclonal antibodies cross-react with various Actinobacteria, including *Nocardia* spp., *Tsukamurella paurometabolum*, *Streptomyces* spp., as well as *Candida albicans* found in normal mouth flora. Cross-reactions with environmental non-tuberculous mycobacteria (NTM) are less likely to play a role as Qvist *et al.*⁽⁷⁷⁾ have recently demonstrated low cross-reactivity of the Determine™ LAM LFA assay with NTM in a study evaluating the clinical utility of the urine LAM LFA in a cystic fibrosis cohort. Although the numbers were small, we had five participants from whom NTM were cultured from sputum. Only one of these participants had positive urine LAM LFA.

The low sensitivity of the Determine™ LAM LFA may be explained by multiple factors. One of these is that circulating LAM molecules are often complexed with anti-LAM antibodies and these immune complexes are too large to pass through the intact glomerular membrane into the urine.^(75, 78) In addition, Sakamuri *et al.*⁽⁴⁷⁾ demonstrated that LAM is complexed to lipid carriers in the serum (e.g. high-density lipoprotein) and this may further account for false negative results if the LAM is not dissociated from the lipid carrier prior to entering the kidney. The abovementioned explanations however are unlikely to solely explain the low sensitivity in our study as one would expect these antibodies would be present in the blood of most patients with TB. However, there may be varying ratios of antibodies to antigen in each individual patient, with immunocompromised patients having lower antibody levels and thus this may explain the higher sensitivity of the assay in patients with CD4 T-cell counts <100 cells/mm³.

Another explanation for the decreased sensitivity of the assay is that false negative results may be due to levels of LAM excretion in the urine below the level of detection of the LAM LFA. Urine LAM is excreted in variable concentrations between 100 picograms/mL to several hundred nanograms/mL.^(31, 42, 50, 79, 80) Savolain *et al.*⁽⁸⁰⁾ showed that a 100-fold concentration step increased the sensitivity of the Clearview™ TB ELISA in the HIV-negative group with TB.

Another possibility is the variability of LAM production by various strains.^(35, 81) Mutations which affect the structure of LAM have been identified in clinical strains of *M. tuberculosis*.⁽⁸²⁾ Torelles *et al.*⁽⁸²⁾ described two clinical strains with truncated and more branched forms of LAM. It is possible that mutated forms of LAM may not bind to the polyclonal antibody incorporated into the LAM LFA. Additionally, sensitivity may be impacted by several other mechanisms including the characteristics of the test-capture antibody and the proportion of urine LAM derived from either renal or extra-renal TB sources.⁽⁷⁵⁾ Tests that incorporate polyclonal antibodies for LAM antigen capture are more likely to recognize multiple antigenic epitopes of LAM compared with monoclonal antibodies targeted at a single epitope.⁽⁸³⁾ However, polyclonal antibodies increase the risk of “batch to batch” variation during the manufacturing process.⁽⁸⁴⁾

Another factor possibly influencing the sensitivity of the urine LAM LFA is the site of TB infection.⁽⁷⁵⁾ The urine LAM LFA is likely to have a lower sensitivity in patients with PTB without associated EPTB in comparison to patients with disseminated TB. Some authors have postulated that in the presence of circulating anti-LAM immunoglobulin, LAM detected in urine might be more likely to reflect local renal involvement with TB rather than distant pulmonary disease.⁽⁴³⁾ Wood *et al.*⁽⁴³⁾ provide some supportive evidence for this observation in their study using the Xpert®MTB/RIF assay

on urine. Approximately half of urine LAM-positive patients were Xpert® MTB/RIF assay positive and none of the urine LAM-negative patients with HIV and confirmed PTB co-infection were urine Xpert®-positive.⁽⁴³⁾ Since the assumption is that the Xpert®MTB/RIF assay detects whole *M. tuberculosis* bacilli⁽¹⁴⁾, these authors⁽⁴³⁾ have suggested that positive results must indicate the presence of *M. tuberculosis* bacilli in the renal tract. However, a recent publication⁽⁸⁵⁾ using heat-inactivated bead-beaten bacilli has shown that the Xpert®MTB/RIF assay is also able to detect DNA from non-viable bacilli, so the hypothesis of Wood *et al.*⁽⁴³⁾ may be incorrect.

5.1.1 Sensitivity in hospitalised versus outpatients

Our study showed that the sensitivity of the LAM assay was higher in hospitalised patients versus outpatients (44.2%; 95% CI [34.0, 54.8] vs. 14.1%; 95% CI [7.5, 23.4]). Similar to our study, Lawn *et al.* reported a sensitivity of 38.1% in hospitalised patients in a South African district hospital.⁽¹¹⁾ Peter *et al.*⁽⁸⁶⁾ reported a sensitivity of 46% (95% CI [39.0, 53.0]) and a specificity of 96.0% (95% CI [82.0, 99.0]) using a grade 2 cut-point of the urine LAM LFA in a hospitalised HIV-infected population. In an additional study using a grade 2 cut-point, Peter *et al.*⁽⁸⁷⁾ reported a sensitivity of 48.0% (95% CI [39.0, 57.0]) in hospitalised patients with suspected HIV/TB co-infection and a specificity of 85.0% (95% CI [77.0, 94.0]). In their systematic review, Shah *et al.*⁽⁷²⁾ found that the pooled sensitivity and specificity were 53% (95% CrI [38.0, 70.0]) and 90% (95% CrI [73.0, 96.0]), respectively [four studies; 1,299 HIV-infected inpatients; 514 (40%) with TB].

Drain *et al.*⁽⁷¹⁾ reported that amongst HIV-infected participants presenting to outpatient clinics in South Africa, when using LAM LFA grade ≥ 2 as indicative of a positive result, one LAM test had a sensitivity of 41% (95% CI

[28.0 , 55.0]) and a specificity of 89% (95% CI [85.0, 93.0]) for culture-confirmed TB. This is in contrast to our study where we reported a much lower sensitivity (14.1%, 95% CI [7.5, 23.4]) in the outpatient population. It is possible that the patients recruited in the aforementioned study were more immunosuppressed than our participants in the outpatient group, hence accounting for the lower sensitivity reported in our study. In support of this hypothesis is that fewer outpatient participants in our study had CD4 T-cell counts ≤ 100 cells/mm³ [15.8% (46/291) in our study vs. 23.3% (67/288) in the study by Drain *et al.*⁽⁷¹⁾ ($p=0.02$)]. In support of our findings of low sensitivity of the Determine™ LAM LFA assay in the outpatient HIV-infected population, Peter *et al.*⁽⁵⁹⁾ reported in the TB-NEAT study that “in African HIV-TB co-infected outpatients able to self-expectorate sputum, LAM had limited sensitivity even at low CD4 counts, and offered no significant incremental diagnostic yield over Xpert®MTB/RIF or smear microscopy”.

5.1.2 Smear-negative participants

5.1.2.1 Sensitivity and specificity

Amongst smear-negative participants, the sensitivity and specificity of the urine LAM LFA was 22.0% (95% CI [14.7, 31.0]) and specificity was 98.7% (95% CI [96.7, 99.7]) when compared to mycobacterial culture as a reference method. Peter *et al.*⁽⁵⁶⁾ reported that the sensitivity of the urine LAM LFA in smear-negative/sputum-scarce participants in their study of hospitalised participants with suspected TB/HIV co-infection was 39.0% (95% CI [28.0, 52.0]). In another study, Peter *et al.*⁽⁸⁶⁾ demonstrated that the urine LAM test was positive in just over half of their HIV-infected hospitalised participants with advanced immunosuppression and smear-negative, culture-positive TB.

5.1.2.2 Incremental sensitivity over smear

In our study, the urine LAM LFA detected 22% (24/109) of smear-negative, culture-positive participants. We demonstrated that the urine LAM LFA detected 14% (8/57) of smear-negative, culture-positive participants in the outpatient setting and 30.8% (16/52) of smear-negative, culture-positive hospitalised participants. Drain *et al.*⁽⁷⁰⁾ reported that the urine LAM test identified 10% more cases of culture-positive PTB than sputum AFB microscopy in their initial study of HIV-infected outpatients presenting to an antiretroviral clinic. In a second study, Drain *et al.*⁽⁷¹⁾ reported that a single LAM LFA identified an additional 26% of participants with culture-confirmed or clinically suspected PTB, as compared with AFB microscopy alone ($p<0.05$). In their systematic review and meta-analysis, Minion *et al.*⁽⁵²⁾ found that urine LAM tests were positive in 41% of smear-negative specimens; this was a relatively large incremental yield compared with smear microscopy. This suggests that the LAM assay and smear microscopy may detect different groups of TB patients.⁽⁵²⁾

Although the sensitivity of the Determine™ LAM LFA test seems modest, the high specificity makes the test a potentially useful rule-in test in approximately 1/5th of participants in our study that would have been missed by smear microscopy alone. Peter *et al.*⁽⁸⁶⁾ reported that 25 of 39 (64%) patients with positive urine LAM results would also have been missed by smear microscopy in their study on hospitalised HIV-infected patients in Cape Town. The assay therefore shows potential as a useful adjunctive test for the diagnosis of TB particularly in resource-poor settings where newer NAATs such as the GeneXpert® MTB/RIF assay are unlikely to be utilised.

5.1.3 Sensitivity and specificity stratified by CD4 T-cell count

The sensitivity of the Determine™ urine LAM assay was highest in those participants with CD4 T-cell counts ≤ 100 cells/mm³ particularly those with CD4 counts ≤ 50 cells/mm³ (Figure 11). This is similar to the findings reported by other authors evaluating the Determine™ kit as well as various other LAM assays.^(11, 40, 43, 48, 52, 56-58, 71, 72, 88) As mentioned previously, Wood *et al.*⁽⁴³⁾ reported the sensitivity of LAM LFA in HIV-infected patients ($n=146$) with CD4 T-cell counts of ≥ 200 , 100-199, 50-99, and < 50 cells/ μ l, was 15.2%, 32%, 42.9%, and 69.2% respectively. Shah *et al.*⁽⁵¹⁾ who used the Clearview LAM ELISA reported sensitivity was 55% (95% CI [41.0, 69.0]) for those with CD4 counts greater than 200 cells/mm³, 71% (95% CI [51.0, 87.0]) for CD4 counts of 50–100 cells/mm³, and 85% (95% CI [73.0, 93.0]) for CD4 counts less than 50 cells/mm³. Drain *et al.*⁽⁷⁰⁾ reported the sensitivity of the Determine™ urine LAM test sensitivity was 37.5% (95% CI [21.1, 56.3]) for those with CD4 T-cell count <100 /mm³, whilst specificity was 86.9% (95% CI [75.8, 94.2]) in comparison to microbiological sputum culture. In a recent Cochrane review⁽⁷²⁾, the authors found that the urine LAM LFA had a pooled sensitivity and specificity of 62% (95% CrI [49.0, 73.0]) and 89% (95% CrI [77.0, 95.0]), respectively amongst participants with CD4 T-cell counts ≤ 50 cells/mm³ (four studies, 467 participants). In contrast, in participants with CD4 T-cell counts >200 cells/mm³, the pooled sensitivity and specificity were 15% (95% CrI [8.0, 27.0]) and 96% (95% CrI [89.0, 99.0]), respectively (four studies, 870 participants).⁽⁷²⁾

Drain *et al.* showed that the diagnostic sensitivity of urine LAM among adults with CD4 T-cell count <100 cells/mm³ was 2.3-fold higher than those with a CD4 T-cell count ≥ 100 cells/mm³ ($p = 0.03$).⁽⁷¹⁾ In our study, having a CD4 T-cell count <50 cells/mm³ was associated with an almost 15-fold increased risk of having a positive urine LAM LFA test result. Lawn *et al.*⁽¹¹⁾

reported that 85.1% (63/74) participants with CD4 T-cell counts <100 cells/mm³ could be diagnosed with urine testing alone. This phenomenon has also been demonstrated using the Xpert®MTB/RIF assay on urine.⁽⁸⁹⁾ Lawn *et al.*⁽⁸⁹⁾ reported a diagnostic yield of 44.4% in participants with CD4 counts <50 cells/ μ L compared to the yield of 2.7% in participants with CD4 counts >100 cells/ μ L when performing the Xpert®MTB/RIF assay on urines ($p=0.001$). When using the Clearview™ ELISA LAM, Shah *et al.*⁽⁹⁰⁾ reported that the median quantitative urine LAM test result was over 10-fold higher than the current threshold for test positivity in those with CD4 counts <50 cells/mm³ and 5-fold higher than this threshold in those with CD4 counts between 51 and 100. This phenomenon is likely due to higher mycobacterial loads in patients with severe immunosuppression (impaired antimycobacterial immune response), and hence higher levels of LAM antigen excreted in urine.^(48, 50, 51, 90, 91) In support of this, Schwebach *et al.*⁽³⁵⁾ showed that the amount of surface-mannosylated outer membrane component, arabinomannan, produced during growth of *M. tuberculosis in vivo* is dependent on the bacterial number per infected organ. Another explanation is that “due to the general lack of cavity formation in immunosuppressed patients, the bacteria replicate within tissue” instead of the cavity and this may further “facilitate the diffusion of shed LAM” into the circulatory system.⁽⁵²⁾ A further explanation is that there is a lower degree of antigen-antibody complex formation (lack of high avidity immunoglobulin) in HIV-infected patients with immune suppression and this may facilitate LAM excretion in the urine.^(29, 43) Patients with lower CD4 T-cell counts are also more likely to have lower albumin levels⁽⁹²⁾, hence there is more free LAM in the circulation as less is bound to lipid carriers like albumin. Finally, immunosuppressed patients are more prone to have disseminated TB and the urine LAM result may reflect renal tract TB as part of this presentation.⁽⁴³⁾ These findings suggest the urine LAM LFA [as opposed to the existing TB diagnostics (smear microscopy, radiology and NAATs)], whose performance is impaired with greater degrees of

immunosuppression, is most useful in patients with advanced immunodeficiency.

5.2 Cut-points for reading of strips

We found using ROC analysis that a Grade 2 cut-point maximised sensitivity and specificity of the LAM LFA assay. Specificity using a Grade 2 cut-point was 98.7% (95% CI [96.7, 99.7]) versus Grade 1 [76.6% (95% CI [71.5, 81.2]). Similarly, Peter *et al.*⁽⁵⁶⁾ reported that when the test strips were used at a “Grade 1 cut-point as recommended by the manufacturer” initially, “specificity in culture-negative patients was very poor [66%, (95% CI [57.0, 74.0]) but improved to 96% (95% CI [89.0, 100.0]) using the Grade 2 cut-point.” In contrast, Lawn *et al.*⁽⁵⁵⁾ studying ambulatory patients screened prior to starting antiretroviral therapy (ART) found very high specificity (98.6%; 95%CI [97.0, 99.5]) using a cut-point of Grade 1. These findings have not been replicated by other investigators and may be related to rigorous reading of the strip by a single experienced individual. Reading of the urine LAM LFA at the Grade 1 cut-point is difficult and is unlikely to be a pragmatic expectation of non-laboratory trained HCWs in a busy clinic environment. Our data therefore lend support to an expert panel’s decision to use the Grade 2 cut-point as a diagnostic threshold.^(61, 66)

5.3 Inter-reader correlation

We found a good correlation between different readers using a Grade 2 cut-point ($k=0.9$; 95% CI [0.8, 0.9]). Peter *et al.*⁽⁵⁶⁾ found the inter-reader correlation was moderate ($k=0.8-0.9$) when using a Grade 2 cut-point. Lawn *et al.*⁽⁵⁵⁾ reported extremely high inter-reader agreement of $k=0.97$. In another

study, Peter *et al.*⁽⁵⁶⁾ reported notable differences in inter-reader agreement when performing the Determine™ urine LAM assay in HIV-infected hospitalised patients with severe immunosuppression. They found disagreement was due to misinterpretation of the very faint band (Grade 1 band).⁽⁶⁶⁾ Agreement improved when using a Grade 2 band as cut-point.⁽⁶⁶⁾

5.4 Clearview™ LAM ELISA

We found that the Clearview™ LAM ELISA had a sensitivity and specificity of 38.9% (95% CI [31.7, 46.4]) and 99.7% (95% CI [98.2, 100.0%]) respectively, in comparison to the Determine® urine LAM LFA assay which had an overall sensitivity of 30% (95% CI [23.4, 37.3]) and a specificity of 98.7% (95% CI [96.7, 99.7]). Of the 30 participants who were Clearview™ urine ELISA-positive, but urine LAM LFA-negative, all were confirmed TB culture-positive. The reported sensitivities and specificities of the Clearview™ urine LAM ELISA vary from 13-93% and 88-97%.^(52, 57, 93) Similar to our study, Gounder *et al.*⁽⁹⁴⁾ reported that the sensitivity and specificity of the urine LAM ELISA compared to the gold standard of positive bacteriology or histopathology were 32%(95% CI [16.0, 52.0]) and 98% (95% CI [96.0, 99.0]) respectively in a cohort of 422 HIV-infected participants presenting to a clinic at Tembisa, South Africa. Shah *et al.*⁽⁵¹⁾ reported the overall Clearview™ LAM test sensitivity was 59% (95% CI [52.0, 66.0]) in participants with confirmed TB, and the specificity was 96% (95% CI [91.0, 99]) among individuals without TB.

In general, the Clearview™ TB ELISA appears to be more sensitive than the urine LAM LFA.^(55, 87) Peter *et al.*⁽⁸⁷⁾ found that the Determine™ urine LAM LFA had a sensitivity of 48.0% (95% CI [39.0, 57.0]) compared to 58.0% (95% CI [49.0, 67.0]) with the Clearview™ TB ELISA in a cohort of HIV/TB co-

infected hospitalised patients. Lawn *et al.*⁽⁵⁵⁾ showed a good correlation between Clearview™ LAM ELISA and LAM LFA in their study. However, Nicol *et al.*⁽⁶⁰⁾ showed a poor agreement between the LAM LFA and the Clearview™ ELISA in a paediatric study conducted in South Africa. The variability of performance of the assay in these different studies may be attributed to batch variations of the LAM LFA assay or methodology of performing the test (i.e. frozen versus fresh urine).

5.5 LAM LFA results and mycobacteraemia

Our study revealed that 65.2% (30/46) of participants with a positive blood culture had a positive urine LAM LFA test. This reinforces the findings of Shah *et al.*⁽⁹⁰⁾ who similarly demonstrated a strong association between urine LAM detection and mycobacteraemia. Nakiyingi *et al.*⁽⁹⁵⁾ reported urine LAM positivity in 29 (70.7%) of the 41 HIV-infected smear-negative participants with positive blood cultures in their study in Uganda. In contrast, Talbot *et al.*⁽⁸⁸⁾ did not find higher sensitivity in patients with positive blood cultures in a study evaluating the Chemogen LAM ELISA in HIV-infected participants in Dar-Es Salaam with symptoms of TB. Unlike other studies^(95, 96), we found that 67.4% (31/46) participants with positive blood cultures were sputum smear-positive (either by ZN or auramine staining).

Nakiyingi *et al.*⁽⁹⁵⁾ reported that CXRs were reported as abnormal and suggestive of TB in 62% of their patients with mycobacteraemia. We found 85.4% (35/41) participants with mycobacteraemia had CXRs reported as highly suggestive or compatible with TB. In our study, the combination of smear and chest radiography would have diagnosed a significant proportion of patients with mycobacteraemia.

5.6 Incremental value of combination of diagnostic tests

5.6.1 Urine LAM LFA combined with smear microscopy

Our study showed that the sensitivity and specificity of combining urine LAM LFA and ZN smear microscopy was 52.8% (95% CI [45.2, 60.3]) and 98.7% (95% CI [96.7, 99.7]). This confirms the findings of other investigators that the combined sensitivity is greater than either test alone.^(50, 51, 56, 57, 72) A recent Cochrane review⁽⁷²⁾ reported a pooled sensitivity of a combination of LAM LFA and sputum microscopy (either test positive) was 59% (95% CrI [47.0, 70.0]), which represented a 19% (95% CrI [4.0, 36.0]) increase over sputum microscopy alone, while the pooled specificity was 92% (95% CrI [73.0, 97.0]), which represented a 6% (95% CI [1.0, 24.0]) decrease from sputum microscopy alone (four studies; 1,876 participants; 38% with TB).

Drain *et al.*⁽⁷⁰⁾ found that when the urine LAM LFA was combined with sputum microscopy (either test positive), sensitivity increased to 38.3% (95% CI [26.0, 51.8]), but specificity decreased to 85.8% (95% CI [81.1, 89.7]). These authors found that the addition of urine LAM to sputum microscopy detected an additional 20% of culture-positive PTB cases ($p=0.02$), though at the expense of identifying an additional 9.6% as false positives.⁽⁷⁰⁾ Peter *et al.*⁽⁵⁶⁾ reported a combined smear microscopy and urine LAM LFA sensitivity of 71% (95% CI [62.0, 78.0]) and specificity of 73% (95% CI [65.0, 80.0]). Shah *et al.* found that when compared with the yield from sputum smear fluorescence microscopy alone, additional use of the urine LAM assay increased the sensitivity from 42% to 67%.⁽⁹⁷⁾

In another multicentre outpatient study, Peter *et al.*⁽⁵⁹⁾ found that the combined sensitivity of urine LAM LFA and sputum microscopy was 56.2% (95% CI [45.9, 66.5]) and specificity was 89.6% (95% CI [85.3, 93.9]). Lawn *et al.*⁽⁵⁵⁾ similarly found that the urine LAM LFA provided incremental diagnostic yield when combined with sputum microscopy in an HIV-infected adult outpatient setting. The authors found that the incremental sensitivity of urine LAM LFA and TB microscopy in combination (either test positive) attained a sensitivity of 72.2% (95% CI [46.5, 90.3]) in participants with CD4 T-cell counts <50 cells/ μ L.⁽⁵⁵⁾ The same authors⁽¹¹⁾ found in another study that additional use of the urine LAM assay increased the diagnostic yield 2.5-fold when compared with the yield from fluorescence microscopy of sputum smears alone.

Dheda *et al.*⁽⁴⁸⁾ showed in their study evaluating the Clearview™ urine LAM assay, that in the group of participants with CD4 T-cell counts <200 cells/mm³, smear microscopy and urine LAM ELISA had non-redundant overlap, i.e. the two tests identified different patient groups. This may account for the reported increased sensitivity when the tests are combined.

5.6.2 Urine LAM LFA combined with smear microscopy versus Xpert[®] MTB/RIF assay or in combination with the Xpert[®]

Shah *et al.*⁽⁹⁷⁾ found that the combination of smear microscopy (two sputum specimens) and LAM LFA identified 67% (69/103; 95% CI [0.57, 0.76]) of culture-confirmed TB cases and approached sensitivity of Xpert[®] testing alone (76%, 95% CI [0.66, 0.84]; $p = 0.15$). These authors found the combined sensitivity of the Xpert[®] and LAM LFA was 85.4% (88/103; 95% CI [77.0, 92.0]), which was superior to either test alone ($p < 0.05$) and approached sensitivity of sputum liquid culture testing (94%, 95% CI [88.0, 98.0],

$p=0.17$).⁽⁹⁷⁾ Kroidl *et al.*⁽⁷⁶⁾ demonstrated a combined sensitivity of 60% (smear microscopy and LAM LFA) and 90% (Xpert® and any LAM test positive (LFA/ELISA) in a paediatric study. Lawn *et al.*⁽⁵⁵⁾ in the study described previously found the incremental sensitivity of urine LAM LFA and TB microscopy in combination (either test positive) was comparable to the sensitivity of a single Xpert® on sputum in HIV-infected outpatients with CD4 counts <50 cells/ μ L. The same authors found in another study⁽¹¹⁾ that compared with the yield from testing sputum with the Xpert®MTB/RIF assay, additional use of urine LAM assay increased the diagnostic yield twofold.

Similar to other researchers, our data supports the use of the LAM LFA test as a useful add-on test for rapid diagnosis of TB in HIV-infected patients. Combination of urine LAM with Xpert®MTB/RIF assay may improve performance of TB diagnostic testing algorithms. These combinations have already been shown to be cost-effective.^(98, 99)

5.7 Chest radiography

The sensitivity and specificity of chest radiography compared to culture-confirmed TB as a reference was 78.1% (95% CI [70.9, 84.1]) and 59.9% (95% CI [53.6, 65.9]), respectively. When combined with a urine LAM LFA test, the sensitivity and specificity were 81.7% (95% CI [74.9, 87.3]) and 59.5% (95% CI [53.2, 65.5]), respectively. The addition of smear microscopy to CXR and urine LAM LFA improved sensitivity only slightly (82.3%, 95% CI [75.6, 87.8]). Padmapriyadarsini *et al.*⁽¹⁰⁰⁾ reported that CXR had a sensitivity and specificity of 72% (95% CI [60.0, 82.0]) and 57% (95% CI [55.0, 59.0]), respectively, when compared to TB culture in their study of 492 HIV-infected ambulatory patients with smear-negative TB. Peter *et al.*⁽⁸⁶⁾ reported 85%

(95% CI [79.0, 89.0]) sensitivity and 30% (95% CI [16.0, 49.0]) specificity of CXR in their study on HIV-infected hospitalised patients. These authors however did not comment on how and by whom the radiographs were read.⁽⁸⁶⁾

Lawn *et al.*⁽⁸⁹⁾ reported that the “positive predictive value of Determine™ TB-LAM for TB was found to be high” amongst “those with radiographic abnormalities” and thus suggested that “combined testing would increase diagnostic specificity in those with abnormal radiographs”. In contrast, we found only 27 (22.7%) participants with an abnormal CXR highly suggestive of TB had a positive urine LAM LFA and 16 (14.2%) participants with an abnormal CXR that may be compatible with TB had a positive urine LAM LFA. In our study, only seven (3.7%) of 191 participants with normal radiographs or abnormal CXRs not due to TB were urine LAM LFA-positive. Interestingly, of these participants, six (85.7%) were confirmed TB culture-positive. It appears that CXR and urine LAM LFA may detect different subsets of patients with TB. This also lends support to the hypothesis that the urine LAM LFA detects renal tract TB as opposed to PTB (which may be detected by CXR).

The LAM assay may be of use in facilities lacking radiology services or could assist in guiding empiric anti-TB therapy in the subgroup of patients with CXRs that may be compatible with TB whilst awaiting confirmation from other diagnostic modalities.

5.8 Correlation of urine LAM LFA and proteinuria

Seventeen (25%) of our participants with proteinuria $\geq 300\text{mg/dL}$ had a positive urine LAM test whereas only 6.1% (13/213) of participants with no

proteinuria had a positive urine LAM test (Z score 4.39; $p < 0.05$). Reither *et al.*⁽⁵⁴⁾ reported a significant positive association with the Chemogen LAM ELISA and proteinuria. Proteinuria was independently associated with LAM positivity in a paediatric study conducted in Tanzania.⁽⁷⁶⁾ In comparison, Dheda *et al.*⁽⁴⁸⁾ showed that there was no association between urine LAM positivity and proteinuria [6/24 (25%) who did have proteinuria vs. 2/12 (17%) who did not have proteinuria]. Wood *et al.* reported mild to moderate proteinuria in both urine LAM-positive and LAM-negative subjects.⁽⁴³⁾ They did not report any heavy proteinuria (sufficient to cause leakage of immune-complexed LAM into the urine) in any of the urine LAM LFA-positive subjects.⁽⁴³⁾

There are two possible explanations for the proteinuria seen in our participants with positive urine LAM LFAs. HIV-infected patients may develop a number of renal abnormalities ranging from HIV-associated nephropathy (HIVAN), HIV-related immune complex disease, nephropathy secondary to antiretrovirals or antibiotics, thrombotic microangiopathy, and diseases related to common comorbidities such as hepatitis C virus-related membranoproliferative glomerulonephritis.⁽¹⁰¹⁾ HIV-infected patients of African descent appear to be more susceptible to HIVAN.⁽¹⁰²⁻¹⁰⁴⁾ It is therefore possible that the proteinuria detected in our participants reflected underlying HIV-associated renal disease. Regrettably, protein-creatinine ratios or serum creatinine levels were not obtained on our participants. These measurements are more accurate estimations of proteinuria and excretory functions of the kidney than a urine dipstick and could have confirmed or refuted this hypothesis. A second explanation is that the proteinuria reflects underlying renal tract TB. Peter *et al.*⁽⁸⁷⁾ similarly found a strong association between LAM in the urine, proteinuria and increasing urine Xpert®MTB/RIF positivity. A protein/creatinine ratio >0.03 g/L was associated with a 6.2-fold (95% CI [2.3, 17.1]) odds of having a positive

Xpert® ($p < 0.001$).⁽⁸⁷⁾ More studies are required to elucidate the biological and pathological mechanisms of proteinuria in this subset of patients.

5.9 LAM LFA results and outcomes

Wood *et al.* reported that LAM positivity was not associated with worse TB treatment outcomes.⁽⁴³⁾ In contrast, other authors have shown an association with worse outcomes.^(59, 105-110) Manabe *et al.*⁽¹⁰⁹⁾ found that LAM or cryptococcal latex agglutination test positivity in hospitalised patients in Uganda was associated with subsequent death within two months. These authors reported that 21.4% (75/351) of their study population demised within two months; 50.7% (38/75) of whom were TB LAM-positive. The median CD4 T-cell count of their participants was 57 (IQR 14-179) cells/mm³. A recent systematic review and meta-analysis⁽¹¹¹⁾ of the detection of urine LAM as an independent predictor of mortality in patients receiving treatment for HIV-associated TB in sub-Saharan Africa found that case fatality rates varied between 7% and 53% (10 studies; 1,172 patients). Pooled summary estimates generated by random-effects meta-analysis showed a twofold increased risk of mortality for urinary LAM-positive HIV-TB cases compared to urinary LAM-negative HIV-TB cases (relative risk 2.3, 95 % CI [1.6, 3.1]).⁽¹¹¹⁾ The authors also found that, of five studies which reported multivariable analyses of risk factors for mortality, pooled summary estimates demonstrated an over twofold increased mortality risk (OR 2.5, 95 % CI [1.4, 4.5]) among urinary LAM-positive HIV-TB cases, even after adjustment for other risk factors for mortality, including CD4 T-cell count.⁽¹¹¹⁾ A recent randomised control trial⁽¹¹⁰⁾ of urine LAM LFA to guide TB treatment initiation in HIV-infected hospitalised patients conducted in four African countries (South Africa, Tanzania, Zimbabwe and Zambia) found that the urine LAM test used as an adjunctive test reduced all cause eight-week mortality in patients with HIV, symptoms of TB, and advanced

immunosuppression (absolute reduction of 4% (95% CI [1.0, 7.0])). These findings raise the possibility that a positive urine LAM LFA may be a marker for more severe culture-positive disease.

5.10 WHO Policy guidance

Utilising the results of this study as well as additional studies in the field, the WHO formulated a policy guideline on the use of the urine LAM LFA for the diagnosis and screening of active TB in people living with HIV at the end of 2015.⁽⁶⁵⁾ This guideline⁽⁶⁵⁾ has the following recommendations:

1. “The LAM LFA may be used to assist in the diagnosis of TB in HIV-infected adult *inpatients* with signs and symptoms of TB (pulmonary and/or extrapulmonary) who have a CD4 T-cell count ≤ 100 cells/ μ L, or HIV-infected patients who are seriously ill (definition based on 4 danger signs: respiratory rate >30 breaths/min, temperature $>39^{\circ}\text{C}$, heart rate >120 beats/min, and unable to walk unaided) regardless of CD4 count or with unknown CD4 count (conditional recommendation; low quality of evidence).
 - a. This recommendation also applies to HIV-infected adult *outpatients* with signs and symptoms of TB (pulmonary and/or extrapulmonary) who have a CD4 T-cell count ≤ 100 cells/ μ L, or HIV-infected patients who are seriously ill regardless of CD4 count or with unknown CD4 count, based on the generalisation of data from inpatients.
 - b. This recommendation also applies to HIV-infected children with signs and symptoms of TB (pulmonary and/or extrapulmonary) based on the generalisation of data from adults while acknowledging very limited data and concern regarding low specificity of the LAM LFA in children.”

Importantly, the WHO guideline specifies that “except as specifically described above for persons with HIV infection with low CD4 T-cell counts or who are seriously ill, LAM LFA should not be used for the diagnosis of TB” (strong recommendation, low quality of evidence).⁽⁶⁵⁾ In addition, the LAM LFA “should not be used as a screening test for TB” (strong recommendation, low quality of evidence).⁽⁶⁵⁾

6. STRENGTHS

Our study adhered to the recommendations of the STARD (Standards for Reporting of Diagnostic Accuracy Studies) Initiative as well as the QUADAS tool for quality assessment of diagnostic studies. We maintained a rigorous reference standard of culturing two sputa (induced if necessary) as well as blood to detect disseminated TB. We assessed the assay across two sites (Cape Town and Uganda) and across two different clinical settings (outpatient and hospitalised patients). In addition, clinical, laboratory and data management was performed according to Good Clinical Practice standards.

7. LIMITATIONS

We included only well categorised patients with culture-confirmed TB and those in whom active TB was ruled out using a combination of microbiological and clinical data. As such, the sensitivity of the urinary LAM assays could not be assessed for individuals in whom a final TB diagnosis could not be confirmed by culture. Despite liquid culture of sputum and blood, and additional solid culture of sputum, the sensitivity and specificity of the Determine™ urine LAM LFA may have been underestimated. The

overall sensitivity of TB culture is 80-85%⁽¹¹²⁾, hence it is not the ideal reference test. Ideally, a single error-free reference test should be used to determine the final diagnosis and estimate the accuracy of a test.⁽¹¹³⁾ Sputum culture particularly in HIV-infected individuals is an imperfect gold standard test and non-differential misclassification may have led to reduced estimates of diagnostic accuracy. One method to reduce this bias is to use a fixed rule to combine several imperfect tests into a composite reference standard.⁽¹¹³⁾ The combination of several test components would have provided a better perspective on disease status than any individual test alone and accuracy estimates of a test under evaluation will therefore be less biased.⁽¹¹³⁾ In our study, the use of the Xpert® on sputum specimens in addition to the liquid culture of sputum and blood would have reduced this bias.

The Determine™ urine LAM LFA was not performed directly on voided urine at the clinic but rather performed on the same day as collection at a university laboratory. Mukundan *et al.*⁽¹¹⁴⁾ have reported that LAM spiked into urine samples tends to deteriorate after two hours. This phenomenon may have affected the sensitivity of the assay in our study. In addition, the test was read by laboratory technicians who are more likely to report consistent results compared to HCWs at the bedside or clinic, so this may have overestimated the sensitivity of assay as well as inter-reader agreement.

Our study did not evaluate the urine LAM LFA against newer diagnostic standards such as the Xpert®MTB/RIF assay on sputum. This test was not part of standard of care at the time the study was conducted and the Xpert® is known to offer reduced utility in extrapulmonary and sputum-scarce TB. Regrettably, urine mycobacterial culture as well as Xpert®MTB/RIF assay on urine was not performed due to financial constraints.

We did not perform routine urine bacterial culture in our study. It has been hypothesised that bacterial contamination by faecal flora from the perineum could cross-react and potentially account for false positive urine LAM LFAs.

The results of our study are only generalizable to HIV-infected African populations and must be confirmed in other geographical locations.

We did not evaluate clinical outcomes associated with LAM positivity, nor did we evaluate the urine LAM LFA for treatment monitoring response. Ethambutol is known to block lipoarabinomannan synthesis.⁽¹¹⁵⁾ Several studies have suggested that urine LAM may have a role for monitoring response to treatment.^(43, 76) Drain *et al.*⁽¹⁰⁷⁾ found that urine LAM positivity and grade decreased during anti-TB therapy. Kroidl *et al.*⁽⁷⁶⁾ demonstrated a decrease in signal positivity of the Chemogen LAM ELISA at different time points in 13 of 14 paediatric participants on TB treatment.

8. CONCLUSION

In keeping with the recent WHO guideline policy⁽⁶⁵⁾ on the urine LAM LFA, our study has shown that the assay is useful in selected patient populations, particularly hospitalised HIV-infected patients with CD4 T-cell counts <200/ μ L. Based on our findings in the ambulatory patient population, the test should not be used to investigate unselected patients at primary care level. The test offers many advantages: it is a true POC test that can be used by trained HCWs in peripheral clinics or remote settings without laboratory infrastructure or electricity; it is cheap and rapid; specimen collection is easy; the specimen type poses less of a biosafety risk to HCWs; and the urine LAM assay has potential for detection of extrapulmonary TB as well as being a

possible biomarker for response to TB therapy. Despite the sensitivity of the Determine™ urine LAM LFA assay being moderate at best, the use of this test as a rule-in test may allow early treatment initiation and reduce mortality in a subset of patients. In addition, the urine LAM could easily be incorporated into an integrated diagnostic management algorithm for TB. The overall high specificity of the assay, its modest cost, and the lack of rapid diagnostic options lends support to this possibility. Future studies should evaluate the impact of the test on patient-relevant outcomes within an integrated algorithm rather than simple descriptions of test accuracy.

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CHAPTER FOUR: DIAGNOSTIC PERFORMANCE OF THE XPERT[®] MTB/RIF ASSAY ON BLOOD SPECIMENS FROM HIV-INFECTED PARTICIPANTS WITH SUSPECTED TUBERCULOSIS

1. INTRODUCTION

The Xpert[®] MTB/RIF assay (Cepheid, Sunnyvale, California, USA) has played a significant role in revolutionising modern diagnosis of tuberculosis (TB). This automated molecular test is a single-tube, molecular beacon-based real-time polymerase chain reaction (PCR) assay which is able to detect *Mycobacterium tuberculosis* deoxyribonucleic acid (DNA) whilst simultaneously detecting mutations associated with rifampicin (RIF) resistance directly from sputum specimens.⁽¹⁾ The test takes only two hours to perform, potentially allowing patients to be diagnosed and commenced on treatment in the same clinic visit.⁽¹⁾ It is more sensitive than conventional smear microscopy.⁽²⁾ On this basis, the World Health Organisation (WHO) made a recommendation that the Xpert[®] MTB/RIF test be used as the initial rapid diagnostic test for people living with HIV who have suspected pulmonary TB (PTB) as well as for patients with suspected multidrug-resistant (MDR) TB.⁽³⁾

Although PTB is the main form of the disease, disseminated TB is common amongst HIV-infected individuals. Rugina *et al.*⁽⁴⁾ reported disseminated TB in 38% ($n=42$) of their HIV-infected cohort in Romania. A Ugandan study revealed that 23% ($n=86$) of HIV-infected patients hospitalised with severe sepsis had *M. tuberculosis* bacteraemia.⁽⁵⁾ Post-mortem studies have also

highlighted the significant burden of disseminated TB in the HIV-infected population. Martinson *et al.*⁽⁶⁾ confirmed disseminated TB as a cause of death in 60% ($n=47$) of the post-mortems performed on HIV-infected hospitalised individuals with a premorbid diagnosis of TB presenting to two hospitals in Soweto, South Africa. Ansari *et al.*⁽⁷⁾ demonstrated that TB was the leading cause of death in a series of 128 post-mortems performed in 1997 to 1998 in Botswana with 90% ($n=40$) of the TB cases having disseminated extrapulmonary disease.

The diagnosis of disseminated TB can often be challenging, particularly in countries where laboratory services are limited. Diagnosis is predominately based on clinical grounds or microbiological culture of fluids or tissues obtained from extrapulmonary sites.⁽⁸⁾ The absence of typical TB symptoms makes the clinical diagnosis difficult, and the paucibacillary nature of the disease often affects the sensitivity of smear microscopy. In addition, sputum specimens are often difficult to obtain in patients. Feasey *et al.* reported that only 81% ($n=104$) of their participants were able to produce a sputum specimen in their cohort study of consecutive HIV-infected patients presenting with cough and fever to a hospital in Malawi.⁽⁹⁾ TB culture is usually delayed due to the slow replication rate of the bacterium. The classic radiographic manifestations associated with disseminated TB are often missing.⁽¹⁰⁻¹²⁾ Of great concern is that the disease is associated with a high mortality especially in the HIV-infected population.^(5, 10, 11, 13-16) This is partly due to the delay in diagnosis and initiation of antituberculosis therapy. Alternate diagnostic techniques are urgently required to facilitate early diagnosis and reduce mortality. The potential use of a PCR-based test, like the Xpert®MTB/RIF assay, to rapidly diagnose disseminated TB, is an attractive solution to the diagnostic challenges of this condition.

The majority of published studies using PCR-based technologies for diagnosing extrapulmonary or disseminated TB usually include small numbers of extrapulmonary specimens. Sensitivities vary considerably from 3% to 100%.⁽¹⁷⁻¹⁹⁾ In addition, the specimen type varies from pleural fluids, cerebrospinal fluids (CSF), tissues and abscess or lymph node aspirates, making it difficult to compare the different PCR-based tests available. A recent systematic review and meta-analysis⁽⁸⁾ of 18 studies involving Xpert®MTB/RIF assay testing of 4,461 specimens revealed a pooled sensitivity of 83.1% for lymph node aspirates versus culture, 80.5% for CSF and 46.4% for pleural fluid, respectively. The WHO has recently recommended the Xpert®MTB/RIF assay for the diagnosis of TB in lymph nodes and other tissues as well as the preferred initial test for the diagnosis of tuberculous meningitis.⁽³⁾

Several studies conducted in sub-Saharan Africa have shown a high rate of mycobacteraemia in febrile patients presenting to hospital.^(10-12, 20) We hypothesised that this mycobacteraemia could be detected by the Xpert®MTB/RIF assay performed on blood specimens. Testing of blood specimens is an attractive alternative particularly in patients who are unable to expectorate or who have extrapulmonary disease. It also poses less of a respiratory biohazard to laboratory workers. Banada *et al.*⁽²¹⁾ at the Center for Infectious Diseases, New Jersey Medical School, had developed a novel blood lysis-centrifugation method to detect *M. tuberculosis* in large volume blood specimens in conjunction with the Xpert®MTB/RIF assay. The initial study using *Mycobacterium bovis* BCG spiked blood specimens of 10, 5, 1, and 0.25 colony forming units per millilitre (CFU/mL) revealed sensitivities of 100%, 100%, 83%, and 57%, respectively.⁽²¹⁾ The best results were obtained with acid-citrate dextrose solution B as an anticoagulant.⁽²¹⁾ The lysis buffer used in the method was found to be stable at room temperature and at 4°C for up to two months.⁽²¹⁾ Of note, the blood could be stored for up to eight days without a loss of sensitivity of the assay.⁽²¹⁾

Following on the initial success of these experiments, we sought to evaluate the sensitivity and specificity of the Xpert®MTB/RIF assay on blood specimens collected from HIV-infected participants suspected of having TB.

2. AIMS

The main aims of the study were to determine:

- 2.1 the sensitivity and specificity of Xpert®MTB/RIF testing of blood specimens from HIV-infected patients with suspected TB in comparison to a reference standard of liquid mycobacterial culture of sputum and blood;
- 2.2. the failure rate of the Xpert®MTB/RIF assay, expressed as the proportion of Xpert®MTB/RIF tests that require repeat testing due to an indeterminate initial result;
- 2.3 the time to diagnosis, expressed in hours, of positive Xpert®MTB/RIF tests versus positive mycobacterial blood cultures; and
- 2.4 the correlation (if any) between positive Xpert®MTB/RIF testing on blood specimens and CD4 T-cell count.

3. METHODOLOGY

This prospective study was nested within another study entitled ‘Feasibility of Using the Inverness Lateral Flow Urine LAM Test for Diagnosis of Tuberculosis in HIV-Positive TB Suspects in Cape Town, South Africa’. This prospective study was mainly cross-sectional with limited longitudinal follow-up of certain participants meeting pre-defined criteria, i.e.

participants with a positive urine lipoarabinomannan (LAM) test with no positive mycobacterial TB culture at baseline. These participants had a repeat chest radiograph (CXR) and mycobacterial cultures of sputum and blood at 60 days post- enrolment.

3.1 Clinical sites

Participants were recruited from both hospital (GF Jooste Hospital) and outpatient (Town Two clinic, Khayelitsha) settings in Cape Town, South Africa. GF Jooste is a district level hospital with specialist physician services available. Town Two clinic is a primary care clinic with dedicated HIV/TB services. The number of new cases of TB in 2010 in the Western Cape was 49,819 cases (incidence rate of 885/100,000 population) and the prevalence of HIV in the Cape metropole as per the 2010 antenatal survey was 20.2% (95% CI [15.7, 25.6]).⁽²²⁾ Participants were enrolled between January 2011 and November 2011.

3.2 Participants

3.2.1 Inclusion criteria

In order to be eligible for the study, participants had to fulfil the following inclusion criteria:

- age ≥ 18 years;
- provide informed consent;
- have suspected active TB with any one or more of the following symptoms:

- current cough,
- fever at any time within the preceding four weeks,
- night sweats at any time within the preceding four weeks,
and/or
- weight loss within the preceding four weeks;
- HIV-infected, based on any one or more of the following: written results of a positive HIV antibody test and/or written results of a positive HIV viral load and/or documentation in the medical record of positive HIV status by a treating clinician; and
- willingness and ability to comply with study procedures.

3.2.2 Exclusion criteria

Participants fulfilling the following criteria were excluded from the study:

- age <18 years;
- HIV-uninfected or HIV status unknown;
- antituberculosis treatment taken for greater than two days within the previous 60 days;
- unwillingness or inability to provide a urine sample;
- known chronic pulmonary condition, e.g., asthma, chronic obstructive pulmonary disease, emphysema;
- respiratory distress, defined as respiratory rate of >30 breaths per minute or oxygen saturation <90%; or
- any specific condition that in the judgment of the investigator precluded participation because it could affect a subject's safety.

3.3 Microbiological testing

Two sputa were collected at baseline as well as at two month follow-up (follow-up specimens were only obtained if indicated as outlined previously). Sputum induction was performed if participants were unable to spontaneously expectorate a sputum specimen. Direct as well as concentrated fluorescent acid fast bacilli (AFB) sputum smear microscopy (as per WHO guidelines), Mycobacterial Growth Indicator Tube (MGIT) culture (BACTEC MGIT 960, Becton Dickinson and Company, New Jersey, USA) and Lowenstein-Jensen (LJ) culture were performed on the N-acetylcysteine-sodium hydroxide- treated specimen at the National Health Laboratory Services (NHLS) Medical Microbiology laboratory at Groote Schuur Hospital, Cape Town. The identification and susceptibility of cultured isolates were confirmed using the GenoType® MTBDR*plus* line probe assay (Hain Lifescience, GmbH, Nehren, Germany). Due to financial constraints, the Xpert®MTB/RIF assay was not performed on sputum. In addition, this test was not available as routine standard of care in South Africa at the time the study was conducted.⁽²³⁾

Five millilitres of blood was collected into BacT/ALERT®MP mycobacterial blood culture bottles (bioMerieux, Marcy-l'Etoile, France) as per manufacturer's instruction. Bottles were incubated in a continuously monitored blood culture system (bacT/ALERT®3D Microbial Detection System, bioMerieux, Marcy-l'Etoile, France) at the NHLS laboratory. The identification and susceptibility of cultured isolates were confirmed using the GenoType® MTBDR*plus* line probe assay or, in the event of duplicate isolates from the same participant, an anti-MPB64 antibody assay (Capilia TB-Neo, TAUNS Laboratories, Numazu, Japan).

Twenty millilitres of blood was also collected for Xpert®MTB/RIF testing. This blood was collected in acid-citrate dextrose tubes (Becton Dickinson, Woodmead, South Africa) and analysed immediately or stored at 4° Celsius for no longer than 24 hours before testing.⁽²¹⁾

3.4 Additional testing

In addition to a limited history-taking and clinical examination, all male and non-pregnant female patients had a chest radiograph (CXR) taken at baseline and at follow-up, if indicated. An additional 5-10 mL of blood was obtained for CD4 T-cell enumeration which was performed at the NHLS haematology laboratory at Groote Schuur Hospital.

3.5 Xpert®MTB/RIF assay on blood

The test procedure entailed adding red blood cell lysing solution (proprietary information) at 15% (vol/vol) to whole blood. The specimen was mixed thoroughly by inverting the tubes twenty times and then left to incubate at room temperature for 10 minutes. This was followed by a centrifugation step, where the specimen was centrifuged at 4,000 revolutions per minute (3000xg) for 30 minutes. The supernatant was then discarded and one millilitre of phosphate buffer solution (pH 7.2) was added to the pellet. The pellet was resuspended by vortexing and one millilitre of Xpert®MTB/RIF sample reagent (SR) was added to the specimen. It was vortexed again and left to incubate at room temperature for 15 minutes. Following the incubation period, the specimen was vortexed and then loaded into the

Xpert®MTB/RIF assay cartridge. The standard protocol for the assay was then run as per manufacturer's instruction.⁽¹⁾

3.6 Case definitions

Based on the results of microbiological testing, the participants were categorised into three groups ('Definite TB', 'Probable TB' and 'Not TB') according to strict definitions. The definitions were as follows:

- 'Definite TB' was based on *M. tuberculosis* complex being cultured from a clinical specimen (sputum and/or blood).
- 'Probable TB' was based on any one or more of the following: smear microscopy positive for AFB, but no culture positive for *M. tuberculosis* or other non-tuberculous mycobacteria; caseous necrosis or granulomas on histopathology (if done), and/or clinical response to TB treatment, absence of criteria for 'Definite TB' and absence of a plausible non-TB alternative diagnosis.
- Classification as 'Not TB' was based on the presence of a plausible alternative diagnosis or that the participant did not meet criteria for 'Definite TB' or 'Probable TB'.

As the intention was to investigate the sensitivity and specificity of the Xpert®MTB/RIF assay at the first visit at the point-of-care (as this is the envisioned use of the assay), study follow-up data at two months(including microbiology culture results and record review data) were not included in the definition of 'Definite TB'.

3.7 Data management and statistical analysis

Data was collected on standardised clinical research forms and captured onto a secure database. Statistical analysis was performed using Stata v12 software (Statacorp, College Station, Texas, USA). P-values were calculated using the Chi-square test with Yates correction and Fisher's exact test as appropriate on GraphPad Prism 5 (version 5.0f for Windows, GraphPad Software, La Jolla California, USA, www.graphpad.com) to compare patients on antiretrovirals per site as well as for participant characteristic data (Table 1). The Wilcoxon Mann-Whitney rank sum was used to compare CD4 T-cell count distributions per site. The Shapiro-Wilk normality test was performed to determine if the time to positivity was normally distributed. A two-tailed Z- test for comparing two population proportions was performed for the proportional analyses. The kappa co-efficient was used to determine the level of agreement between the different test assays. For the purposes of this study, the five participants who were classified as "probable TB" were classified as 'not TB' for the sensitivity and specificity analyses.

3.8 Ethical approval

The study received ethics approval from the University of Cape Town Human Research Ethics Committee (HREC Ref 045/2008) as well as Johns Hopkins University School of Medicine Ethics Committee.

The study was reported in conformity with the STARD (Standards for Reporting of Diagnostic Accuracy Studies) Initiative as well as the QUADAS tool for quality assessment of diagnostic studies.

4. RESULTS

4.1 Baseline participant characteristics

Four hundred and ninety-three participants were eligible for the study. Of these, 452 (91.7%) participants had an Xpert®MTB/RIF test performed and 492 (99.8%) had a mycobacterial blood culture taken (Figure 1). Four hundred and ninety-one (99.6%) participants had a CD4 T-cell count enumeration performed. One hundred and eight-nine (41.8%) of 452 participants with Xpert®MTB/RIF testing were hospitalised patients. Baseline characteristics of the two study populations are shown in Table 1.

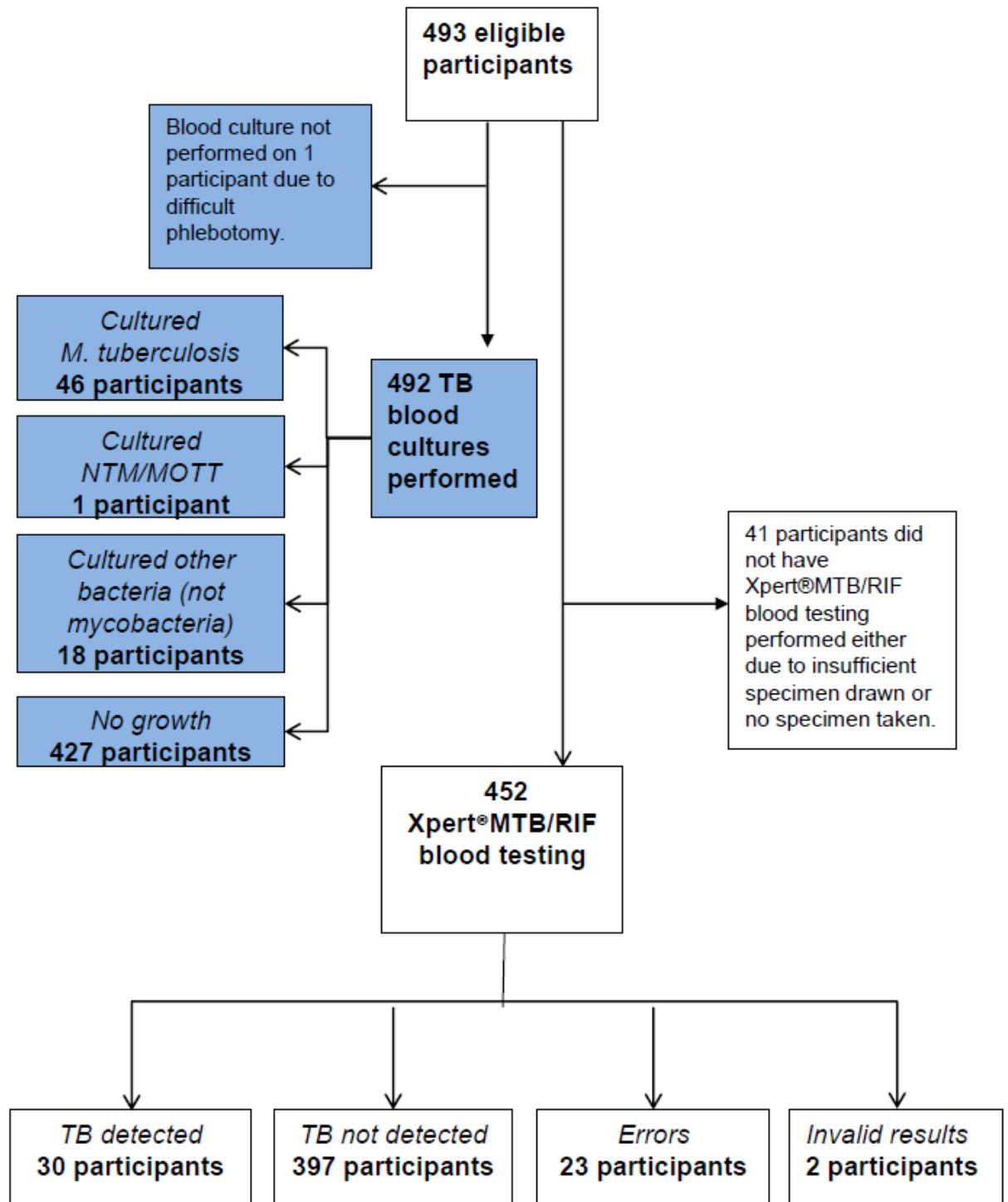


Figure 1. Participant flow diagram.

Key: TB-*Mycobacterium tuberculosis* complex; NTM/MOTT-Non tuberculous mycobacterium/ mycobacterium other than tuberculosis.

Table 1. Baseline characteristics of the study population expressed as frequencies (%).

Participant characteristics	Hospitalised patients (n=189)	Outpatients (n=263)	p-value	Total (n=452)
Demographics				
Male gender	66 (34.9%)	104 (39.5%)	0.367	170 (37.6%)
Median age [IQR]	35 [29-41]	34 [28-41]	NC	35 [28-41]
Presenting symptoms				
Cough*	185 (97.9%)	262 (100.0%)*	0.03	447 (99.1%)
Fever*	147 (77.8%)	186 (71.0%)*	0.21	333 (73.8%)
Night sweats*	138 (73.0%)	255 (97.3%)*	<0.001	393 (87.1%)
Fatigue*	173 (91.5%)	234 (89.3%)*	0.533	407 (90.2%)
Loss of appetite*	152 (80.4%)	246 (93.9%)*	<0.001	398 (88.2%)
Weight loss*	179 (94.7%)	255 (97.3%)*	0.234	434 (96.2%)
Median CD4 T-cell count/μL [IQR]*	121 [48-289.5]	264.5 [153-452]*	<0.001	210 [89-389]
Treatment history				
Currently on antiretrovirals*	78 (41.3%)	47 (17.9%)*	<0.001	125 (27.7%)
Currently on co-trimoxazole prophylaxis*	53 (28.0%)	56 (21.4%)*	0.128	109 (24.2%)

Key: * Outpatient $n=262$ (one outpatient participant did not have a baseline clinical research form completed, therefore symptoms, treatment at baseline and CD4 T-cell count were missing and not included in the analysis above).

The two groups differed in symptoms of cough ($p=0.03$), night sweats ($p<0.001$) and loss of appetite ($p<0.001$) [Table 1]. The hospitalised participants also had a significantly lower median CD4 T-cell count than the outpatient group ($p <0.001$). More participants in the hospitalised group were on antiretroviral (ARV) medication ($p<0.001$).

4.2 Xpert®MTB/RIF testing of blood specimens

Four hundred and twenty-seven (94.5%) of the 452 participants had an interpretable Xpert®MTB/RIF result (Figure 1). There were 23 errors and 2 invalid results. An error is defined as either a probe check failure or a system component failure. Possible reasons for a system component failure include the reaction tube being improperly filled, a problem with reagent probe integrity, the maximum pressure limits being exceeded, or that there was an Xpert® module failure.⁽¹⁾ An invalid result indicates that the sample processing control failed implying that either the specimen was not processed properly or that the PCR was inhibited.⁽¹⁾ Of the interpretable results, 30 participants (7.0%) had a positive Xpert®MTB/RIF test. Of note, twenty-five of the positive results were obtained from hospitalised participants (which accounted 13.2% of the total hospitalised participants) compared to five outpatients with a positive Xpert®MTB/RIF test on blood (1.9% of total outpatients) [$p < 0.001$].

The majority of the positive Xpert® results were very low or low positive (Table 2). Apart from one participant with a RIF indeterminate result, no RIF resistant isolates were detected by Xpert®MTB/RIF testing.

Table 2. Xpert®MTB/RIF semi-quantitative and rifampicin resistance results for participants with a positive result.

Xpert®MTB/RIF result	Number of participants (n)	%
Semi-quantitative Xpert® results	30	100.0
Very low (Ct range >28)*	18	60.0
Low (Ct range 22-28)*	11	36.7
Medium (Ct16-20)*	1	3.3
High (Ct <16)*	0	0
Result for rifampicin resistance	30	100.0
Not detected	29	96.7
Detected	0	0
Indeterminate	1	3.3
No result	0	0

Key: Ct= cycle threshold.

* Ct range obtained from Xpert®MTB/RIF package insert.⁽¹⁾

Four hundred and fifty-one (99.8%) of the 452 participants with Xpert®MTB/RIF tests had mycobacterial blood cultures taken (Table 3). Forty-three (9.5%) participants cultured *M. tuberculosis* complex. Of these, 32 hospitalised participants had a positive blood culture (16.9% of total hospitalised participants), whilst only eleven outpatients (4.2% of total outpatients) had a positive blood culture for *M. tuberculosis* complex ($p<0.001$). Fifteen cultures were positive for other non-tuberculous bacteria.

Table 3. Combined results for Xpert®MTB/RIF and mycobacterial blood culture.

Xpert®MTB/RIF result	Culture-positive for MTB complex	Culture-positive for NTM	Culture-positive for other bacteria	Culture-negative	Total
<i>MTB detected</i>	17	0	0	13	30
<i>MTB not detected</i>	19	0	14	363	396
<i>Error</i>	6	0	1	16	23
<i>Invalid result</i>	1	0	0	1	2
<i>Total</i>	43	0	15	393	451

Key: MTB complex- *Mycobacterium tuberculosis* complex; NTM-non-tuberculous mycobacteria

Of note, the error rate of the Xpert®MTB/RIF assay was 5.5% (25/452). This included the two invalid results that were obtained. The invalid results were probably a result of actual PCR inhibition. A single participant (50%) with an invalid result had a positive mycobacterial blood culture. Of the 23 errors, 18 were pressure aborts, three were sample processing control failures, one was a Probe D check failure and remaining error was due to a signal loss detected in the amplification curve for Probe B. The pressure aborts may have arisen from clotted blood within the cartridge from specimens that were incompletely lysed. Six (26.1%) of the participants with errors on Xpert®MTB/RIF had positive mycobacterial blood cultures.

The sensitivity and specificity of the Xpert®MTB/RIF assay using mycobacterial blood culture as the reference standard is shown in Table 4.

Table 4. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of Xpert®MTB/RIF on blood using blood culture and case definition of “Definite TB” as reference standards.

	Blood culture	Definite TB
Sensitivity (%) (95% CI)	47.2 [30.4, 64.5]	17.1 [11.5, 24.1]
Specificity (%) [95% CI]	96.5 [94.2, 98.2]	98.5 [96.3, 99.6]
PPV (%) [95% CI]	56.7 [40.9, 71.2]	86.7 [69.8, 94.8]
NPV (%) [95% CI]	95.0 [93.3, 96.3]	68.2 [66.6, 69.8]

Key: Positive predictive value (PPV): The probability that a subject with a positive test truly has the disease; Negative predictive value (NPV): The probability that a subject with a negative test truly doesn’t have the disease; CI-Confidence Intervals.

There was moderate agreement between the two test formats ($k=0.473$, $SE=0.049$, $p<0.001$). The sensitivity and specificity of the Xpert®MTB/RIF assay using the case definition of ‘Definite TB’ as reference standard is also shown in Table 4. One hundred and sixty-four of 452 participants met the definition of ‘Definite TB’ with 161 participants having a positive TB sputum culture and three participants with positive blood cultures only (Figure 2).

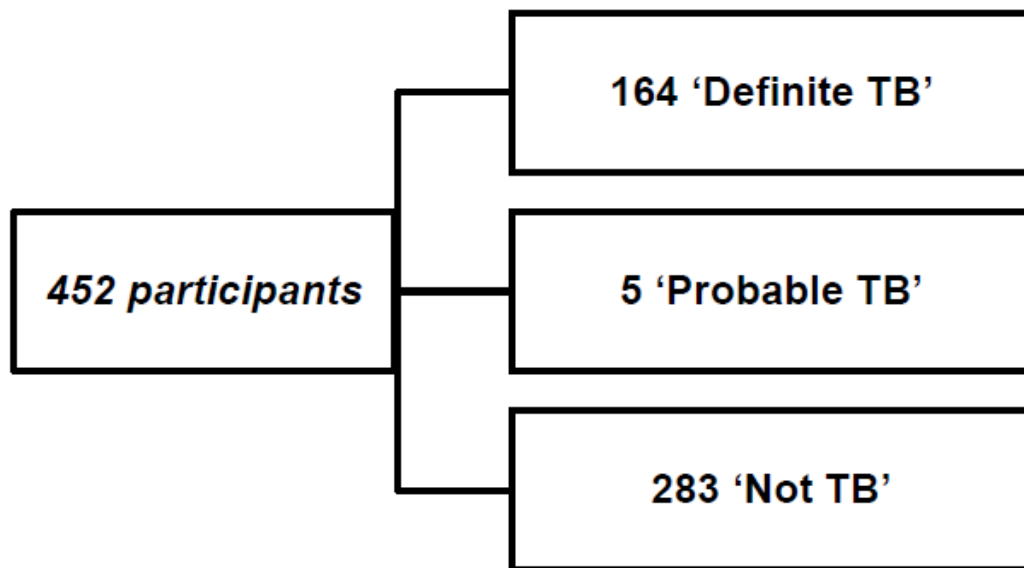


Figure 2. Participants classified as per case definitions.

4.3 Xpert®MTB/RIF test results and smear microscopy

Twenty-four (80%) of the thirty participants with positive Xpert®MTB/RIF blood results were sputum smear-positive (ZN and/or auramine). Twenty seven (90%) of these participants were confirmed culture-positive: 26 (86.7%) had positive mycobacterial culture of sputum, and one (3.3%) of these participants had a positive blood culture only [both MGIT and LJ cultures were contaminated on this participant].

Ten of thirteen (76.9%) of Xpert®-positive, blood culture-negative participants had positive sputum cultures. Interestingly, ten of these thirteen participants were sputum smear microscopy positive [of whom six (60.0%) were 3+AFB positive on concentrated auramine smear microscopy]. Five (83.3%) of these six were 3+ AFB positive on direct ZN staining reflecting a heavy bacterial load of TB.

4.4 Test results and influence of TB treatment

Twenty-three (5.6%) of 412 participants were on TB treatment (<48 hours) at the time of testing. Of these participants, seven (30.4%) were Xpert®-positive, culture-negative; and 15 (65.2%) were both blood culture-negative and Xpert®MTB/RIF-negative (Figure 3). The proportion of patients (8/32) with discordant Xpert®MTB/RIF-positive, culture-negative results who were on TB treatment was statistically significant compared to the proportion of patients (15/380) with concordant results who were on TB treatment ($p<0.05$). A single participant on TB treatment was mycobacterial blood culture-positive, Xpert®-negative.

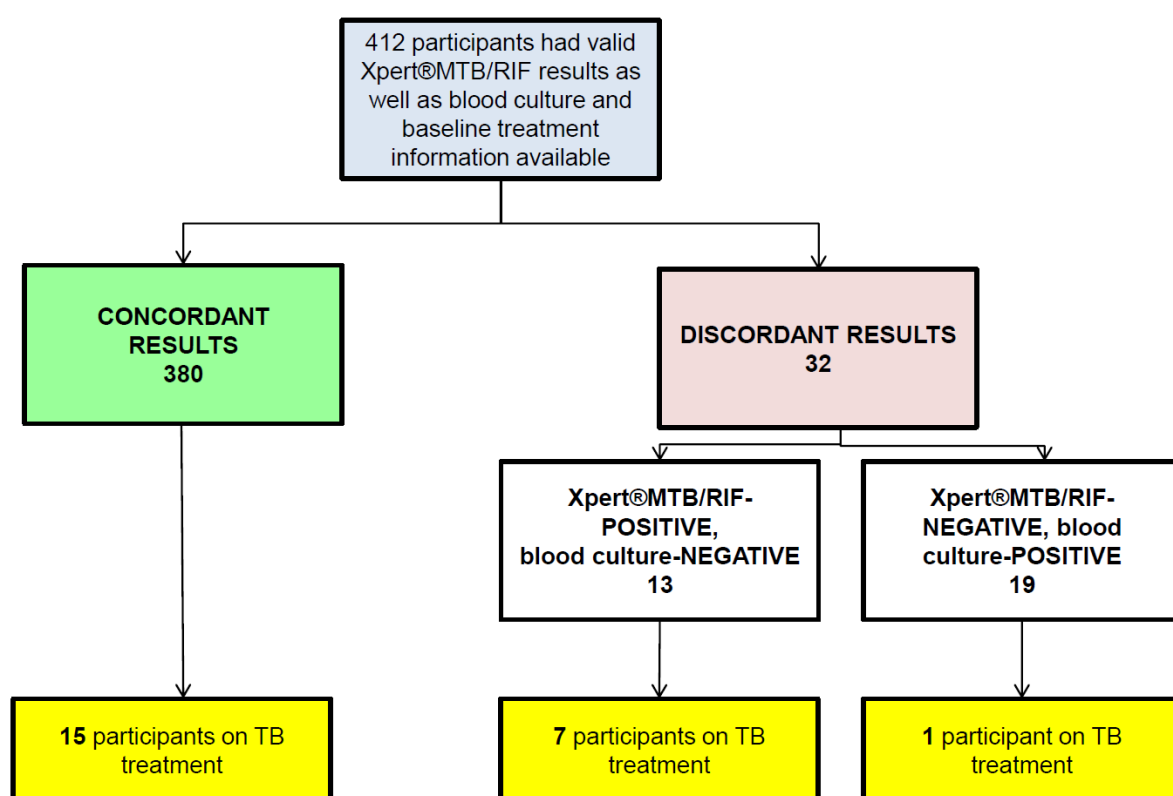


Figure 3. Results of Xpert®MTB/RIF and blood culture testing.

4.5 Time to positivity

Time to positivity was available for 39 (90.7%) of 43 positive blood cultures. The median time to positivity was 476.3 hours [IQR 376.4-627.9; Shapiro-Wilk normality test; $p=0.083$]. This is in contrast to the results of the Xpert® testing which were available within 24 hours after specimen receipt.

4.6 Correlation of Xpert®MTB/RIF test results and CD4 T-cell count

The sensitivity and specificity of the Xpert®MTB/RIF blood test compared to the case definition of 'Definite TB' as stratified according to CD4 T-cell count of the participants is shown in Table 5.

Table 5. Sensitivity and specificity of Xpert®MTB/RIF stratified by CD4 T-cell count category using the case definition of 'Definite TB' as reference standard.

CD4 T-cell count category	Sensitivity (%) [95% CI]	Specificity (%) [95% CI]	PPV (%) [95% CI]	NPV (%) [95% CI]
Total participants [#] (n=426)	17.1 [11.5, 24.1]	98.5 [96.3, 99.6]	86.7 [69.8, 94.8]	68.2 [66.6, 69.8]
<50 cells/μL (n=72)	42.2 [27.7, 58.9]	92.6 [75.7, 99.1]	90.5 [70.6, 97.4]	49.0 [42.3, 55.8]
50-99 cells/μL (n=41)	20.8 [7.1, 42.2]	94.1 [71.3, 99.9]	83.3 [39.0, 97.5]	45.7 [39.9, 51.6]
100-199 cells/μL (n=87)	2.6 [0.1, 13.8]	100.0 [92.8, 100.0]	100.0*	57.0 [55.7, 58.3]
≥200 cells/μL (n=226)	2.2 [0.1, 11.8]	99.5 [97.0, 100.0]	50.0 [6.0, 94.0]	80.4 [79.6, 81.1]

Key: # n=426 [452 participants (total) less 25 participants with error/invalid results. In addition, CD4 T-cell count result was missing for one participant].

* could not calculate confidence interval

4.7 Xpert®MTB/RIF sensitivity and specificity compared to smear microscopy

The relationship between sputum smear microscopy (ZN and concentrated auramine) and blood culture or Xpert® blood testing is shown in Table 6 and 7, respectively. The sensitivity and specificity of blood culture compared to ZN as the reference standard was 29.4% (95% CI [19.0, 41.7]) and 94.0% (95% CI [91.1, 96.2]) respectively, and 30.8% (95% CI [21.5, 41.3]) and 95.8% (95% CI [93.2, 97.7]) respectively when compared to concentrated auramine stained sputum microscopy as the reference. The sensitivity and specificity of the Xpert®MTB/RIF assay compared to ZN stain as the reference was 25.8%

(95% CI [15.5, 38.5]) and 96.2% (95% [CI 93.7, 97.9]) respectively, and 28.9% (95% CI [19.5, 39.9]) and 98.3% (95% CI [96.2, 99.4]) respectively as compared to auramine staining as the reference standard.

Table 6. Xpert®MTB/RIF and blood culture by direct ZN smear expressed as frequencies (%).

DIRECT ZN	BLOOD CULTURE (n =451)		XPRT®MTB/RIF (n =427)	
	<i>Positive</i>	<i>Negative</i>	<i>Positive</i>	<i>Negative</i>
<i>Positive</i>	20 (4.4)	48 (10.6)	4 (0.9)	56 (13.1)
<i>Negative</i>	23 (5.1)	360 (79.8)	26 (6.1)	341 (79.9)
TOTAL	43 (9.5)	408 (90.4)	30 (7.0)	397 (93.0)

NB. Participants with missing microscopy results on the second sputum were classified according to the first sputum smear result. For blood culture, n=451 as one participant was missing blood culture data.

Table 7. Xpert®MTB/RIF and blood culture by concentrated auramine smear expressed as frequencies (%).

CONCENTRATED AURAMINE	BLOOD CULTURE (n =451)		XPRT®MTB/RIF (n =427)	
	<i>Positive</i>	<i>Negative</i>	<i>Positive</i>	<i>Negative</i>
<i>Positive</i>	28 (6.2)	63 (14.0)	24 (5.6)	59 (13.8)
<i>Negative</i>	15 (3.3)	345 (76.5)	6 (1.4)	338 (79.2)
TOTAL	43 (9.5)	408 (90.5)	30 (7.0)	397 (93.0)

NB. Participants with missing microscopy results on the second sputum were classified according to the first sputum smear result. For blood culture, n=451 as one participant was missing blood culture data.

The sensitivity and specificity of both blood culture and Xpert®MTB/RIF assay compared to smear microscopy was increased in the hospitalised patient population (Table 8). The sensitivity and specificity of blood culture compared to ZN stain was 42.1% (95% CI [26.3, 59.2]) and 89.4% (95% CI [83.4, 93.8]) respectively; whilst for the Xpert®MTB/RIF, it was 41.2% (95% CI [24.7, 59.3]) and 92.4% (95% CI [86.7, 96.1]) respectively.

Table 8. Xpert®MTB/RIF and blood culture by ZN sputum microscopy expressed as frequencies (%) for the hospitalised population.

DIRECT ZN	BLOOD CULTURE (n =189)		XPRT®MTB/RIF (n =178)	
	<i>Positive</i>	<i>Negative</i>	<i>Positive</i>	<i>Negative</i>
<i>Positive</i>	16 (8.5)	22 (11.6)	14 (7.9)	20 (11.2)
<i>Negative</i>	16 (8.5)	135 (71.4)	11 (6.2)	133 (74.7)
TOTAL	32 (16.9)	408 (83.1)	25 (14.1)	153 (85.9)

NB. Participants with missing microscopy results on the second sputum were classified according to the first sputum smear result. For the Xpert®, n=178 as 11 hospitalised participants had invalid/error results.

4.8 Xpert®MTB/RIF sensitivity and specificity compared to urine lipoarabinomannan (LAM) lateral flow assay (LFA)

The sensitivity and specificity of the Xpert®MTB/RIF assay was 47.7% (95% CI [32.5, 63.3]) and 97.9% (95% CI [95.9, 99.1]) respectively, when compared to the urine LAM LFA test (which was described in the previous chapter) as the reference test. Using the reference standard of 'Definite TB', the sensitivity and specificity of the urine LAM and Xpert®MTB/RIF were 27.9%

(95% CI [21.2, 35.4]); 98.2% (95% CI [95.5, 99.4]), and 17.1% (95% CI [11., 24.1]); 98.9% (95% CI [96.8, 99.8]), respectively.

5. DISCUSSION

The main aim of our study was to determine the sensitivity and specificity of the Xpert®MTB/RIF assay on blood specimens from participants suspected of having TB. We found that the assay has a sensitivity of 47.2% (95% CI [30.4, 64.5]) and a specificity of 96.5% (95% CI [94.2, 98.2]) when compared to blood culture as a reference method. Using the case definition of “Definite TB” as a reference standard, the sensitivity of the Xpert®MTB/RIF assay decreased to 17.1% (95% CI [11.5, 24.1]) whilst the specificity was 98.5% (95% CI [96.3, 99.6]). Feasey *et al.*⁽⁹⁾ reported a 21% positivity rate using the Xpert®MTB/RIF test on blood using a similar methodology as ours in consecutive HIV-infected participants with fever and cough presenting to Queen Elizabeth Central Hospital in Malawi. In a small study of 24 HIV-uninfected, smear-negative, culture-positive participants, Shenai *et al.*⁽²⁴⁾ reported a sensitivity of only 8.3% (95% CI [2.3, 25.8]) when the Xpert® was performed on blood specimens using the same methodology as our study. Other studies utilising different nucleic acid test formats on blood specimens from patients with suspected TB have reported sensitivities ranging from 18.8% to 95.7%.⁽²⁵⁻²⁸⁾ Dubey *et al.*⁽²⁵⁾ found 17% ($n=130$) of their study population had a positive result whilst evaluating the use of the Blacklight® Card to standardise TB PCR in blood samples using a multiplex nested PCR method targeting insertion sequence (IS) 6110 and sequence of MPB64. Lima *et al.*⁽²⁷⁾ reported PCR sensitivity using a nested IS6110 multiplex PCR on blood was significantly higher in extrapulmonary disease [55.6%, ($n=9$)] than for pulmonary disease [18.2%, ($n=33$)] in a paediatric population suspected of having TB. Our sensitivity (17.1 % using ‘Definite TB’ as the reference method) was similar to Lima *et al.*⁽²⁷⁾ perhaps reflecting more participants in

our study with pulmonary disease than true extrapulmonary/disseminated disease.

One hundred and sixty-four (36.3%) of 452 participants were microbiologically confirmed culture-positive (sputum and/or blood). Forty-three (9.5%) of 451 participants were blood culture-positive. Of these, 32 hospitalised patients had a positive blood culture (16.9% of total hospitalised patients). We detected a low rate of bacteraemia amongst ambulatory patients in our study [11/263 (4.2%)]. A Malawian study⁽²⁹⁾ of 455 HIV-infected out-patients in a programme setting with unexplained chronic fever and/or weight loss and/or chronic diarrhoea similarly detected only 11 participants (2.4%) with positive mycobacterial blood cultures. Monkongdee *et al.*⁽³⁰⁾ similarly had a low yield of two per cent ($n=1,051$) of positive mycobacterial blood cultures in an outpatient study investigating the performance of TB diagnostics in HIV-infected participants in Vietnam and Thailand.

Thirteen participants had positive Xpert®MTB/RIF tests and negative blood cultures. A possible reason for this discrepancy is the lower volume of blood injected into the mycobacterial blood culture bottles in our study which may have affected the yield of culture. We injected a maximum of five millilitres into the blood culture bottle whilst 20 mL (concentrated) was used for the Xpert®MTB/RIF assay. Several studies^(31, 32) have shown that the yield of blood culture is improved by increasing the volume of blood collected. One study showed that the yield of bacteria in blood cultures increases by three per cent for each millilitre of blood cultured.⁽³³⁾ Banada *et al.*⁽²¹⁾ showed similar results for detection of *M. tuberculosis* in blood where five- to tenfold more mycobacterial CFU were required to spike one millilitre blood samples when compared to 20 mL blood samples to achieve Xpert®MTB/RIF test positivity. Another possibility to account for the discordant results is that the

Xpert®MTB/RIF assay detected the DNA of non-viable bacteria that spilled into the blood from substantial localised pulmonary infection. Ten (76.9%) of the 13 participants with positive Xpert®MTB/RIF tests and negative blood cultures had AFB detected on sputum smear microscopy, of which six were 3+ AFB-positive, indicating a heavy burden of disease. Although participants were recruited who had less than 48 hours antituberculosis treatment, a third possibility is that the antibacterial drugs rapidly sterilised the blood, accounting for the Xpert®-positive, but culture-negative results. As described previously, the proportion of patients (8/32) who were on TB treatment with discordant Xpert® results was statistically significant compared to the proportion of patients (15/380) with concordant results who were on TB treatment ($p < 0.05$). This is the most likely explanation to account for the discrepant positive Xpert®MTB/RIF, negative blood cultures results.

Nineteen participants with positive blood cultures had negative Xpert®MTB/RIF tests. The median time to positivity of a blood culture flagging positive in this study was 476.3 hours [IQR 376.4-627.9]. The median time to positivity for blood culture-positive participants with a positive Xpert®MTB/RIF test ($n=15$) and a negative Xpert®MTB/RIF test ($n=17$) was the same: 488.0 hours [IQR 355.8-656] and 488.1 hours [IQR 400.1-643.9], respectively. As per the manufacturer, if the inoculum is $<10^2$ CFU/mL, it would take approximately 319.2-451.2 hours for a bottle to flag positive.⁽³⁴⁾ Crump *et al.*⁽³⁵⁾ have confirmed that the lower the bacterial load, the longer the time to positivity. As the average time to positivity in our study was long, this implies very low concentrations of mycobacteria in the blood ($<10^2$ CFU/mL), possibly at levels below the detection level of the Xpert®MTB/RIF assay which is optimised for detection of mycobacteria in sputum (4.5 (95% CI [3.3, 9.7]) genome copies of purified DNA or 131 CFU/mL (95% CI [106, 176]) of sputum).⁽³⁶⁾ This could have possibly accounted for the discordant Xpert®-negative, culture-positive results. However, although the numbers are small, as the median time to positivity

between positive and negative Xpert®MTB/RIF tests but blood culture-positives was the same, this is unlikely to account for the discrepant results. Another possible reason for false-negative Xpert®MTB/RIF assay results could be related to the presence of inhibitors. However, previous work by Banada *et al.*⁽²¹⁾ has shown that lysis and centrifugation of blood specimens from blood bank or healthy volunteers did not increase levels of PCR inhibitors. Additionally, the Xpert®MTB/RIF assay contains lyophilised *Bacillus globigii* spores which act as an internal sample processing and PCR control.^(1, 37) The internal control is positive when the *B.globigii*-specific probe produces a Ct ≤38 cycles.^(1, 37) Assays that are reported as negative for *M. tuberculosis* and *B.globigii* are reported as invalid.^(1, 37) This was the case in the two invalid results that we obtained in our study where the cycle thresholds for the *B. globigii* were 39.1 and 40 cycles, respectively. Therefore, PCR inhibition is unlikely to be a major factor accounting for the discordant results. Additional research is necessary to elucidate the reasons for the discrepant Xpert®MTB/RIF-negative, blood culture-positive results.

The error/invalid rate in our study was 5.5%. This is similar to error rates reported when the Xpert®MTB/RIF assay was performed on sputum specimens. Cresswell *et al.*⁽³⁸⁾ reported a total error rate of 10.6% (*n*=5,107) in a large multicentre study. In a recent Indian study, Raizada *et al.*⁽³⁹⁾ reported an error rate of 7.2% when performing the Xpert®MTB/RIF test on sputum specimens. Abdurrahman *et al.*⁽⁴⁰⁾ reported an error rate of 3% (*n*=185) on initial Xpert® testing of pooled sputum specimens. Routinely reported data from the NHLS in South Africa has documented average error rates of <3% when the assay was performed on sputum specimens.⁽⁴¹⁾ We hypothesized that error rates would be higher for blood specimens specifically as the Xpert®MTB/RIF assay was designed for sputum specimens. Of note, Feasey *et al.*⁽⁹⁾ reported a very high error/invalid rate (27%) in a study evaluating the Xpert®MTB/RIF assay on blood specimens despite the author using similar methodology as our study. These authors also reported a number of

“pressure-abort” failures with visible clots noted in many of the blood specimens drawn.⁽⁹⁾ We detected only 18 pressure abort failures. Our lower error rate may be attributed to more rigorous phlebotomy technique resulting in less clotted blood in the specimens collected.

Time to positivity was available for 39 out of 43 (90.7%) positive blood cultures. The median time to positivity was 476.3 hours [IQR 376.4-627.9]. This is in stark contrast to the results of the Xpert®MTB/RIF testing which are theoretically available within 2.5 hours after specimen receipt. Prompt initiation of antituberculosis therapy in severely immunocompromised patients has a distinct survival advantage, and therefore the rapid availability of the Xpert®MTB/RIF test results makes it an attractive diagnostic option. With Xpert®MTB/RIF testing becoming more widely available, testing of blood specimens may be particularly useful for sick hospitalised patients. However, given the Xpert®MTB/RIF assay performance on blood, it is unclear whether it will add much clinical value particularly in the context of hospitalised patients where empirical antituberculosis therapy is likely to be started. Theron *et al.*⁽⁴²⁾ commented that in settings where empirical therapy is prevalent practice and rapid, then the use of the Xpert®MTB/RIF assay on sputum was unlikely to improve patient outcomes. In the TB-NEAT study⁽⁴³⁾, of the 68% of participants ($n=154$) with smear-negative, Xpert®-positive TB, 93% were treated with empirical therapy. As over 80% of the participants in our study with positive Xpert® blood results had positive smear microscopy of sputum, the cost and volume of blood required for an Xpert® blood test may not be justifiable. An Xpert®MTB/RIF assay performed on sputum may be more useful in this clinical scenario particularly where rapid susceptibility test results are required.

The Xpert®MTB/RIF assay was most useful in the group of participants with CD4 T-cell counts $<100/\mu\text{L}$, particularly participants with CD4 T-cell counts less than $50/\mu\text{L}$. Feasey *et al.*⁽⁹⁾ also reported a median CD4 T-cell count of $54/\mu\text{L}$ [IQR 48-60/ μL] in the nine participants with positive Xpert®MTB/RIF blood testing in their study of 104 participants suspected of having TB. Patients with CD4 T-cell counts $<100/\mu\text{L}$ are more likely to have extrapulmonary manifestations of TB as well as higher bacterial loads.⁽⁴⁴⁾ In addition, they are more likely to be too ill to expectorate a good quality sputum specimen or undergo sputum induction and thus Xpert®MTB/RIF testing of blood in this population may be very valuable. As the fatality rate of these patients is high, rapid diagnosis and early initiation of therapy is critical to avert mortality. Crump *et al.*⁽¹⁴⁾ reported that 50% ($n=29$) of their study participants in Tanzania with mycobacteraemia died within 36 days of enrolment.

The urine LAM LFA assay performed better than the Xpert®MTB/RIF assay on blood in our study. To the best of our knowledge, this is the first study comparing the two diagnostic tests. Shah *et al.* demonstrated a strong association between urine LAM detection and mycobacteraemia.⁽⁴⁵⁾ As it is hypothesized that LAM is released from metabolically active or degrading mycobacterial organisms into the serum, it is possible that large quantities of LAM are shed into the bloodstream by metabolically active bacteria which are then detected by the urine LAM LFA. In contrast, the amount of bacteria present in blood (and hence DNA) is much lower, and thus possibly below the limit of detection of the Xpert®MTB/RIF assay, thus accounting for the lower sensitivity of the Xpert®.

Feasey *et al.*⁽⁹⁾ found that positive Xpert®MTB/RIF blood tests were highly predictive of early death with 40% ($n=10$) of positive participants dying within two weeks of admission compared to 94 participants with negative

Xpert®MTB/RIF tests. Although our study was not designed to look at mortality outcomes, only eleven (2.2%) participants demised: three participants were culture-positive, whilst eight participants were both culture- and Xpert®MTB/RIF-negative. Of the three participants who were culture- positive, one had a negative Xpert®MTB/RIF assay, one had an error on the Xpert®MTB/RIF assay and the test was not performed for the remaining participant. Nine (81.8%) participants died in hospital. One hundred and twenty-one (26.4%) of the 459 participants in our study were currently on antiretrovirals at the time of testing, which may have accounted for the lower mortality. An additional factor is that the median CD4 T-cell count of our participants was 213/ μ L ($n=424$) versus 94/ μ L ($n=89$) in the study by Feasey *et al.*⁽⁹⁾ reflecting that the Malawian participants had greater immunosuppression than our participants, and hence were more likely to demise from opportunistic infections.

6. LIMITATIONS

We evaluated the use of the Xpert®MTB/RIF assay in HIV-infected participants presenting to an urban clinic and hospital in Cape Town, South Africa. These participants had access to ARV with relatively high CD4 T-cell counts and were therefore likely to have relatively low rates of extrapulmonary or disseminated TB. It is therefore possible that the utility of the Xpert®MTB/RIF blood assay may have been underestimated in this setting and that the assay may have a greater impact in settings with severely immunocompromised HIV-infected patients where there is an increased prevalence of disseminated TB.

In addition, due to financial constraints, the Xpert®MTB/RIF was not performed on participant sputa.

The reasons for the Xpert®-negative, blood culture-positive discordant results were not evident from this study. Further research is required to elucidate the reasons for this discordance.

7. CONCLUSION

Existing tools are not adequate for detecting TB (particularly disseminated TB) in HIV-infected individuals. There is a need for simple tests that can accurately diagnose disseminated TB in patients with HIV co-infection. Xpert®MTB/RIF testing of blood may play a role in the diagnosis of TB, particularly in hospitalised patients with CD4 T-cell counts $<100/\mu\text{L}$. However, given the sensitivity and specificity of the assay, cost and the requirement for specialised equipment as well as a large volume of blood for testing, it is unlikely that Xpert®MTB/RIF testing on blood specimens will contribute much over other diagnostics such as smear microscopy, Xpert®MTB/RIF on sputum and TB culture in resource-limited settings. The assay requires further refinement and adaptation to blood as a specimen type to improve the sensitivity of the assay and thus become a more useful assay for the diagnosis of disseminated TB.

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CHAPTER FIVE: DEVELOPMENT OF AN INTEGRATED SPUTUM COLLECTING/PROCESSING DEVICE

1. INTRODUCTION

Our collaborators at the Centre for Innovation in Global Health Technologies (CIGHT) at Northwestern University, USA, have developed a novel, sensitive and robust system for the detection of HIV deoxyribonucleic acid (DNA)/ ribonucleic acid (RNA) based on an entirely new micro-analytical system utilising paramagnetic particles to move nucleic acids through phase gates to reaction chambers.^(1, 2) Since this system does not rely on microfluidics, it is highly reliable and inexpensive to manufacture (estimated cost US \$0.50 per test). This technology could feasibly be used for point-of-care (POC) diagnosis of tuberculosis (TB) in low-resource settings.

We therefore proposed to adapt this novel low-cost, low-maintenance, integrated micro-analytical system which had previously been developed for HIV, for the detection of *Mycobacterium tuberculosis* in sputum specimens. A key issue for development of the ‘front end’ of this system is the need to ensure protection of the operator from potential exposure to viable *M. tuberculosis* present in the specimen. We therefore aimed to develop a novel integrated specimen collection and processing device which would eliminate the risk associated with operator handling of infectious material, and which would integrate with the proposed micro-analytical system.

The experiments described further in this chapter refer to the development and testing of this sputum collection/processing device.

2. AIMS

2.1 To develop and test a closed 'front-end' specimen collection/processing container which will facilitate the safe and efficient collection and liquefaction of sputum and lysis of *M. tuberculosis* bacilli. This will consist of an integrated sputum collection and liquefaction system which will permit DNA extraction, with minimal risk to laboratory or clinic staff.

2.1.1 Specific objectives

- a) Develop a concept device for the development of prototypes.
- b) Compare various prototypes, with user acceptability studies to determine the most acceptable and appropriate prototype.
- b) Demonstrate proof of concept for collection of sputum specimens.
- c) Validate suitability for end-use.

3. METHODOLOGY

To enable diagnostic testing for TB with this novel POC polymerase chain reaction (PCR) platform, we proposed to replace the routine sputum cup with a collection device which would allow initial processing steps and inactivation of *M. tuberculosis* before the automated instrument transferred

an aliquot of specimen from the collection device to the assay cartridge for DNA isolation and quantitative/real-time PCR.

Our initial concept was to develop a container where the patient would deposit sputum in one compartment of the container which would have a pre-dispensed liquefaction solution stored in a separate compartment. The technician would place the container in a small processing unit which would release the liquefaction solution into the sputum and sonicate the specimen. An aliquot of the disinfected, sonicated solution would then be transferred to the assay cartridge.

The rigid container would have a blister/reservoir (containing liquefaction solution) bonded to a moulded plastic base which contained the access port for depositing the specimen and transferring the aliquot to be tested. The patient would open the cap, deposit a specimen, and replace the cap. The device, shown in Figure 1(a), has the sputum compartment moulded into the base. Alternatively, the sputum cup could be cold-formed from plastic film and bonded to the base, as shown in Figure 1(b). Concept A would be less expensive, but Concept B would transmit ultrasonic energy (required for downstream lysis of *M. tuberculosis*) more efficiently.

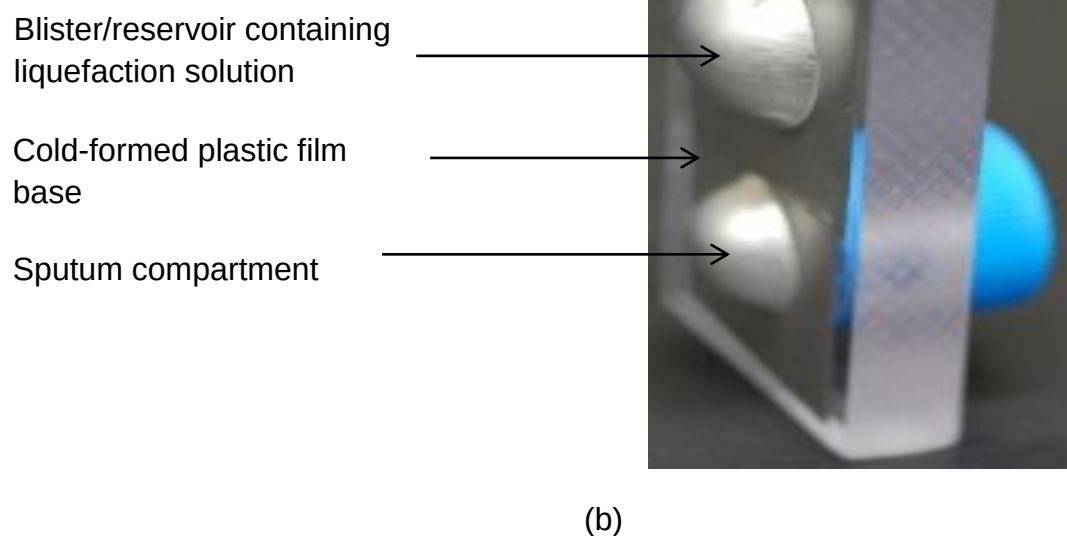
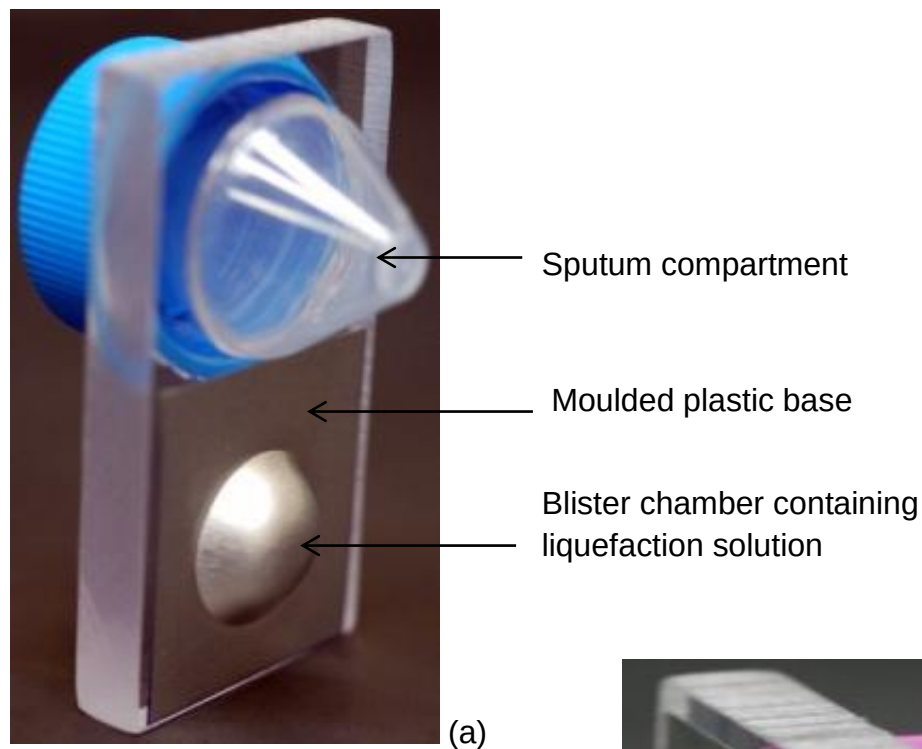


Figure 1. Concept sputum collection devices. (Image courtesy of Dr D. Kelso, Northwestern University).

The device would be loaded into the processor, where the perimeter would be clamped and a plunger would apply pressure to the liquefaction blister, bursting a section of the strip seal, and driving the liquefaction solution into

the sputum compartment (Figure 2). At the same time, an ultrasonic transducer would be brought into contact with the device and energy applied. All of these operations would be done by the operator through a mechanism linked to a lever on the front of the processor. The ultrasonic energy would be set to low levels at first to de-gas and mix the solution, and then higher energy would be applied briefly to disrupt the mycobacterial bacilli. This will have the advantage of not exposing the operator to potentially infectious aerosols. The device would remain closed until after the liquefaction solution had been mixed with sputum and the bacilli disrupted with ultrasonic energy.

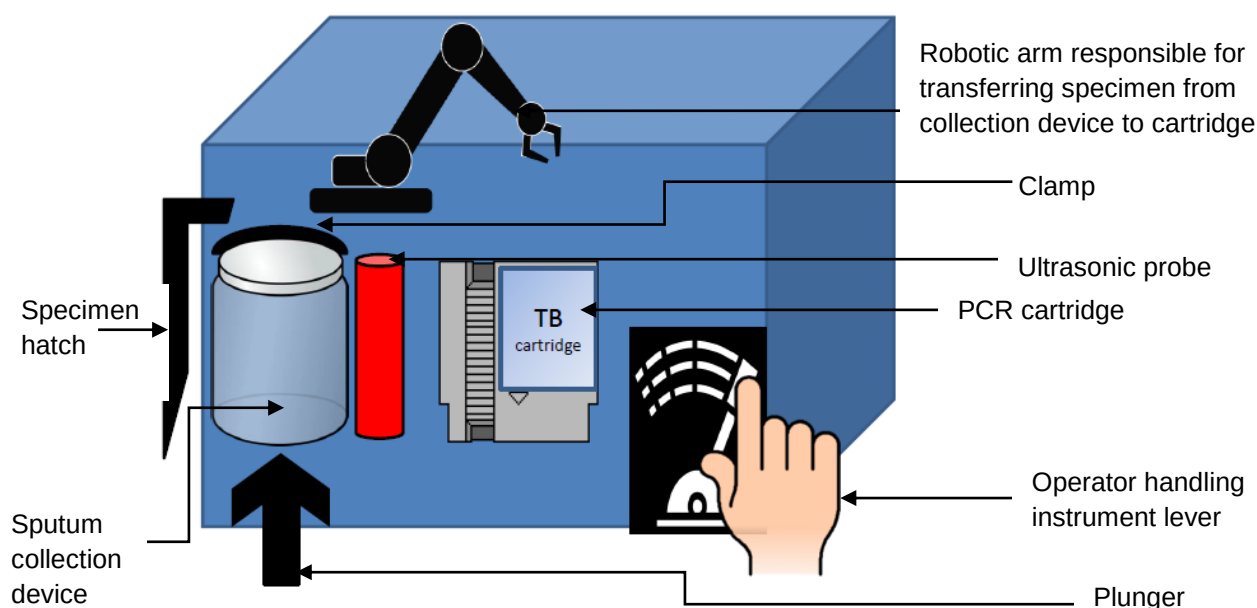


Figure 2. Illustration showing how sputum collection device would integrate into automated PCR machine.

The requirements of the Clinical and Laboratory Standards Institute Laboratory Automation Specimen Container/Specimen Carrier Approved Standard⁽³⁾, recommends the following for any new specimen container that will be used in an automated device:

- “Size 13 x 75 mm, 13 x 100 mm, 16 x 75 mm, or 16 x 100 mm.”
- Container body material should be “glass or plastic, compatible with collection and other processing of specimens for clinical laboratory testing.”
- “Container design should be parallel-walled and usually round-bottom. Flat-bottom tubes or tubes with chamfered bottoms are allowable within the dimensional tolerances specified.”
- “Acceptable closure configurations include:
 - (a) plug closure with or without shields;
 - (b) film seal closure;
 - (c) plug closure integrated with outer guard; or
 - (d) screw-cap closure.”

Our aim was to develop a collection device that met the above standards.

To ensure that the sputum container would be fit for its intended use, we decided that the following characteristics should be tested or inspected to ensure conformance with the device requirements:

1. The average volume of sputum expectorated into the container to ensure that the container met the volume requirements for addition of

liquefaction solution in a 2:1 ratio in addition to the sputum expectorated by the participant.

2. Ease of use of opening and closing the cap.
3. Ease of expectorating into the cup.
4. Robustness of cup to damage during everyday transport.
5. Leakage of specimen (if any).

Three prototypes were developed for further testing and are shown in Figure 3. The first prototype [Figure 3 (a)] was a cylindrical tube with a wide-mouth funnel and a flip-up lid. Sputum would be expectorated into a plastic bag located within the plastic cylinder. The liquefaction solution would be contained within a blister pack attached to the plastic bag. On receipt in the laboratory, the base would be removed and the blister pack would be depressed allow the lysis buffer to mix with the sputum. Prototype 2 [Figure 3(b)] was based on the concept prototype described previously. The patient would deposit a specimen in the first chamber. In the laboratory, the technician would rupture the second chamber allowing the lysis solution to enter the patient specimen chamber. Finally, prototype 3 [Figure 3(c)] was based on the conventional sputum cup with the liquefaction solution contained within a chamber at the base.

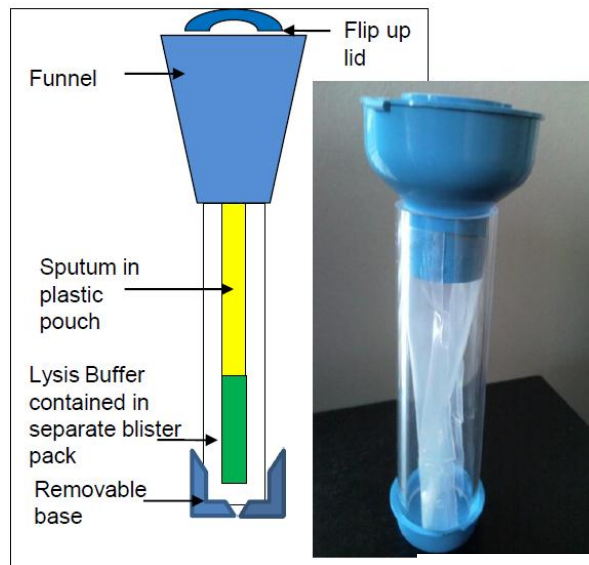


Figure 3(a). Prototype 1

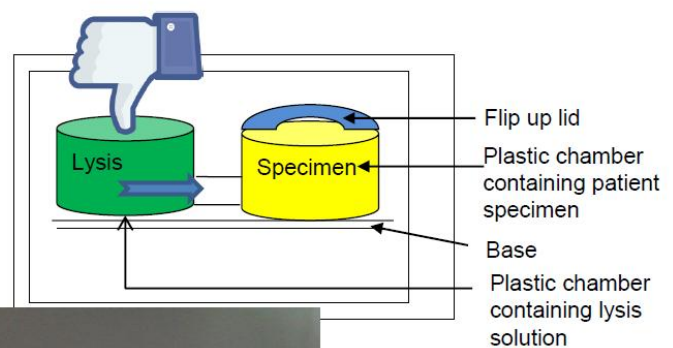


Figure 3(b). Prototype 2

Arrow shows direction of flow of lysis solution once chamber is depressed

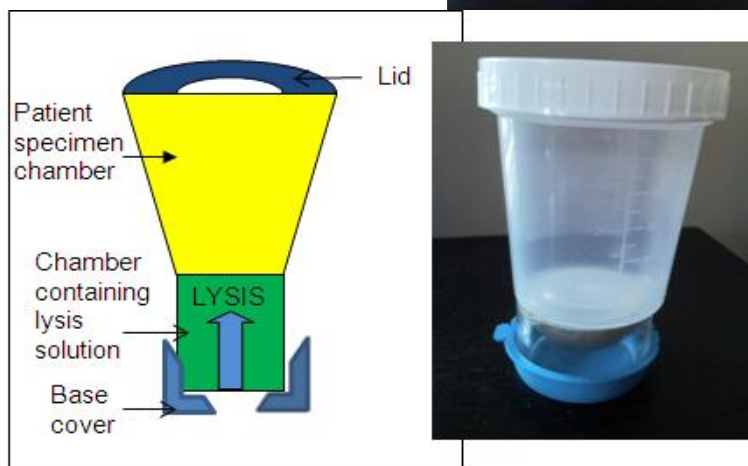


Figure 3(c). Prototype 3

Blue arrow shows direction of flow of lysis solution once chamber is depressed.

Figure 3. The three prototypes (a-c) which were tested during first-phase user acceptability studies.

We conducted two phases of user interface studies to determine the acceptability of the prototype specimen collection/processing device. The first phase evaluated the three prototypes discussed previously (Figure 3), whilst the second phase focused on a refined model of the best candidate prototype selected from the first phase of testing.

3.1 First Phase Testing

The first phase study was conducted by verbal questionnaire in three groups of participants:

1. Randomly selected patients presenting with suspected TB at a primary healthcare clinic (JJ Du Pré Le Roux) in Paarl were approached by the interviewer and verbally asked if they agreed to participate in the survey. Patients who agreed to participate were given a physical model of each prototype and asked to respond to a short series of questions (Appendix 1) regarding the acceptability, ease of use and functionality of the device with regard to sputum collection. The questionnaire did not take longer than twenty minutes to complete. Personal identifying details were not captured during the interview process.
2. Healthcare workers (HCWs) [including registered nurses, patient counsellors]: A similar process was followed for this group of participants, with a slightly amended questionnaire (Appendix 2) to reflect the ease of use of the container from the perspective of clinic staff.

3. Laboratory staff (medical technologists) from the National Health Laboratory Services (NHLS) Medical Microbiology laboratory at Groote Schuur hospital were interviewed (Appendix 3) using the process highlighted above to determine the practicality of the device in terms of existing laboratory procedures.

The first phase of testing was conducted in August 2010 to determine the best prototype out of three possible prototypes that were developed. The prototype was adapted to try to ensure acceptability to all users based on the information gathered from the questionnaires that were administered to participants suspected of having TB, HCWs and laboratory workers. The best prototype was then further developed for second stage testing.

3.2 Second Phase Testing

The second part of this study involved conducting a user interface study to determine the functionality of the final prototype. Following verbal and visual instruction, patients were asked to expectorate into the container to better assess ease of use (Appendix 4). A separate container was provided for each participant and safely discarded after use. The main aim of this second part of the study was to validate sputum collection and transport using the newly designed sputum cup. The following parameters were determined:

- Robustness of the sputum cup: The sputum cup was transported to the University of Cape Town Microbiology laboratory following sputum collection to simulate transit conditions to the sputum microscopy centre/ clinical diagnostic laboratory. This tested container integrity and leakage.

- Leakage testing: This was done on receipt of the specimen by the clinical research worker (CRW) to ensure that the patient had closed the lid of the cup correctly and that the specimen cup was undamaged.
- Assess correct usage: This was performed to ensure that the volume of sputum collected was within acceptable limits for the downstream PCR test, i.e. to ensure that the correct ratio of sputum to liquefaction solution would be contained within the device to ensure optimal results. The sputum cup was given to patients suspected of having pulmonary TB at the clinic. Following instruction by the CRW, the participant expectorated a sputum specimen into the container and the volume of sputum collected was recorded.

This phase of the study was conducted at Town Two clinic, Khayelitsha, where a primary care TB/HIV clinic operates within the municipal clinic. Eligible participants were approached and asked if they agreed to participate in the process. They were asked to provide a third sputum sample which was not used for any routine diagnostic testing. Participants were remunerated a nominal amount for the inconvenience of having to provide a third specimen. Eligibility criteria included that the patient had to be older than eighteen years; willing to give informed consent; capable of producing sputum and able to follow the CRW's instructions to produce a sputum specimen. Participants were instructed on how to produce a good quality sputum specimen (i.e. by repeated deep inhalation and exhalation of breath followed by cough from as deep inside the chest as possible); to produce the correct amount of sputum (to the 2 mL marker on the container); and how to avoid contamination of the exterior of the container (i.e. by carefully expectorating and closing the container) [Appendix 4 and 5].

3.3 Ethical approval

This study received ethical approval by the Health Sciences Faculty Research Ethics Committee of the University of Cape Town (REC REF 154/2009). Informed consent forms were available in three official languages (English, Afrikaans and Xhosa) and the interviews were conducted in the participant's preferred language.

The University of Cape Town as well as Dr David Kelso (Northwestern University, USA) assert joint legal and beneficial ownership of the Intellectual Property arising from this work.

4. RESULTS

4.1 First Phase Study

4.1.1 Patient participant responses

The majority of the participants (15/20, 75%) suspected to have TB felt that Prototype 1 was the easiest container to expectorate sputum into [Figure 4]. Seventeen (85%) participants felt that the flip lid of Prototype 1 was the easiest container to open and close (Figure 5). However, all of the participants thought that the regular sputum container was the easiest to take home to produce a specimen if needed (Figure 6). In summary, 10 of 19 (52.6%) participants thought Prototype 1 was the best container with Prototype 2 being the least favourite (Figure 7). They also generally

commented that they preferred the containers to be smaller (12/20), and more robust (3/20).

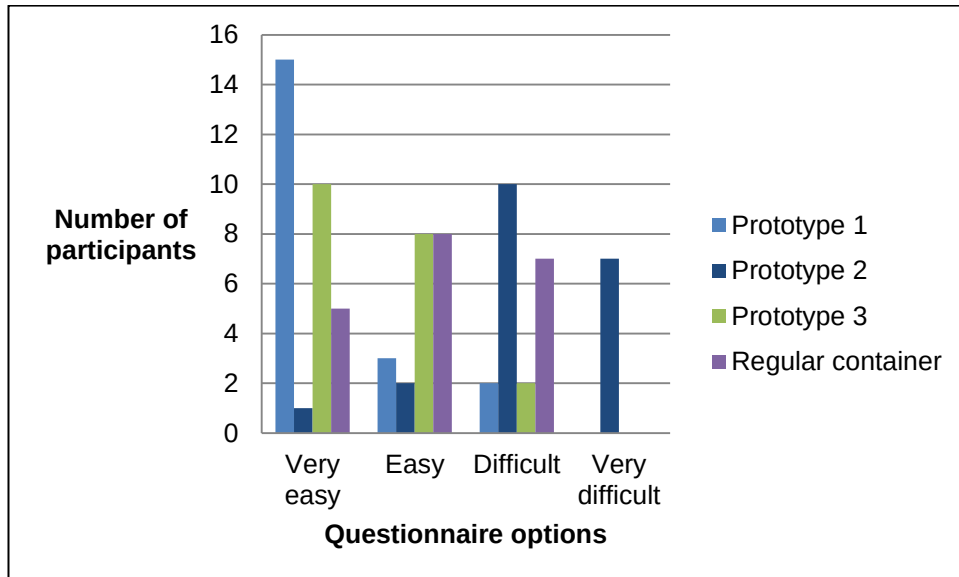


Figure 4. Responses of the participants regarding ease of expectoration into the various prototype devices ($n=20$).

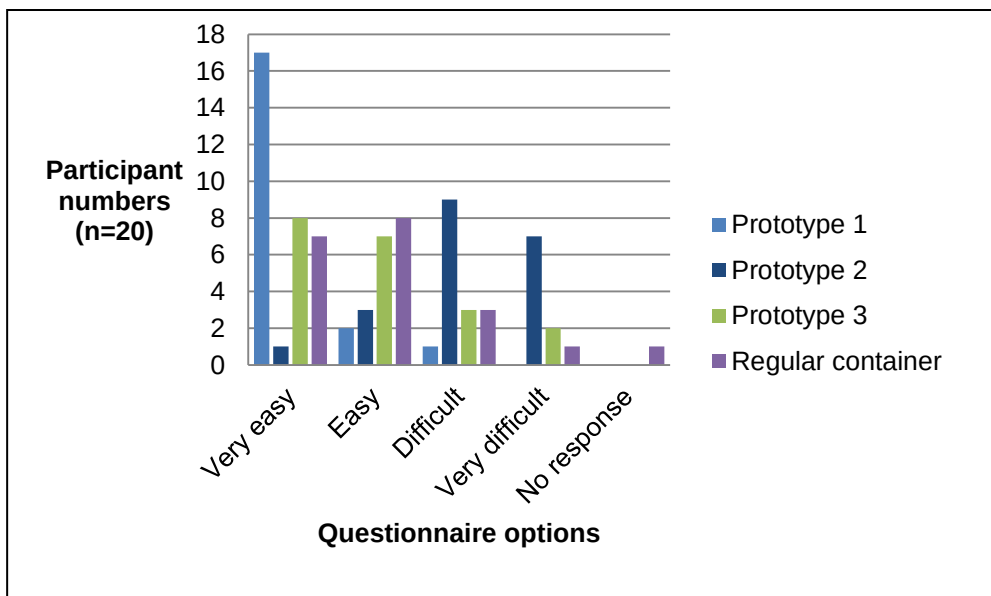


Figure 5. Responses of the participants regarding ease of opening and closing the various sputum containers ($n=20$).

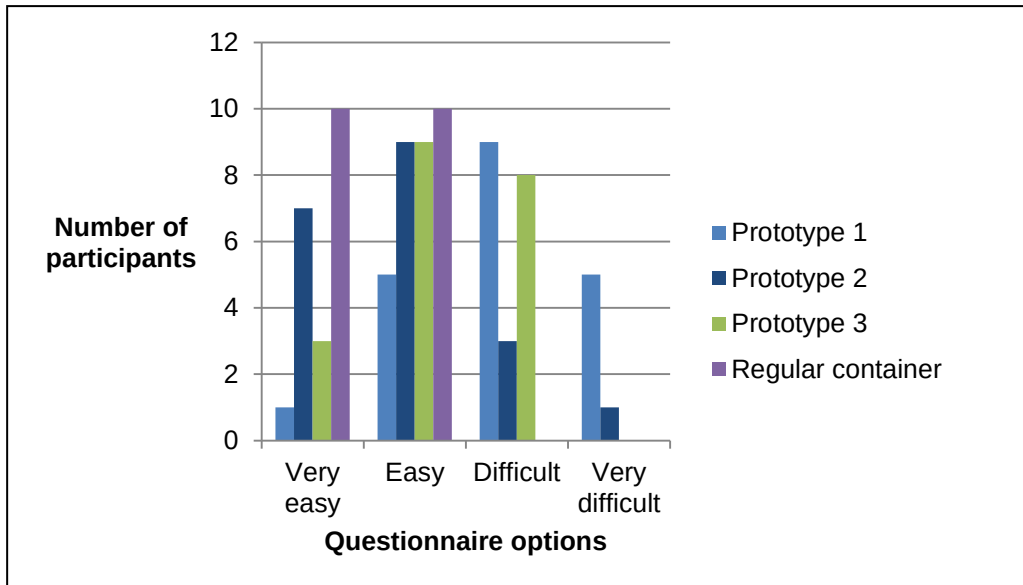


Figure 6. Responses of the participants regarding transporting of sputum container home if necessary ($n=20$).

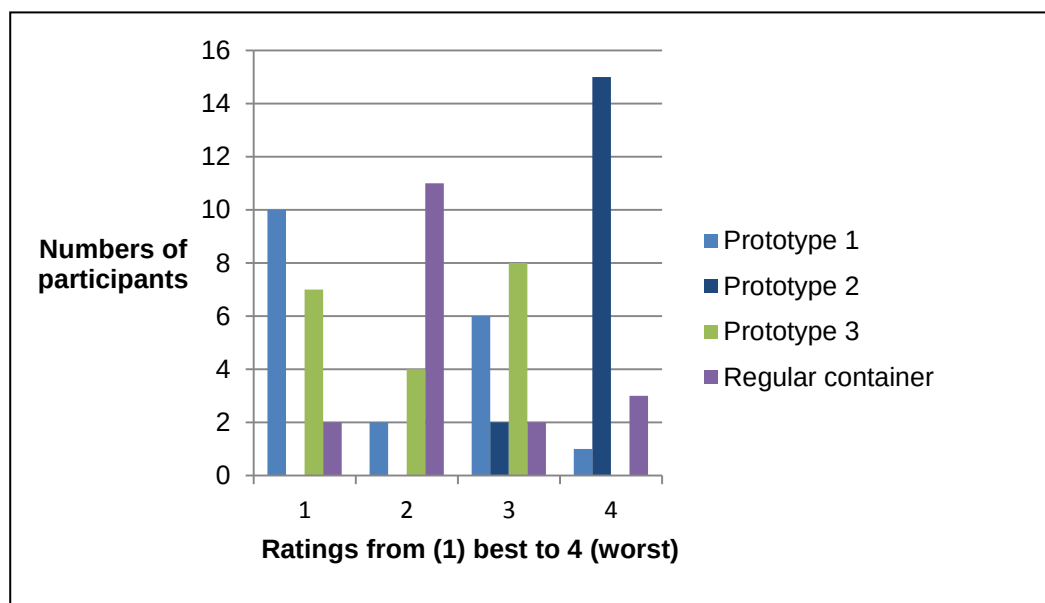


Figure 7. Responses of the participants to which specimen container rated best (1) to worst (4) [$n=19$].

4.1.2 Healthcare worker responses

The healthcare workers (8/10, 80%) believed that the routine sputum cup was the easiest to instruct the patient on sputum collection (Figure 8). Five of the 10 (50%) HCWs interviewed felt that Prototype 1 was the easiest to label. A similar number felt that the regular container was the easiest to transport to the laboratory (Figure 9). In their experience, the majority of HCWs (9/10) thought that between one to two millilitres was the average volume of sputum expectorated by a patient (Figures 10 and 11). Overall, Prototype 1 emerged as the favoured container slightly ahead of Prototype 3 and the regular container, whilst Prototype 2 was the least favoured (Figure 12). Once again, the HCWs commented that the containers should be smaller and fit within the plastic pocket used to transport the specimens to the laboratory.

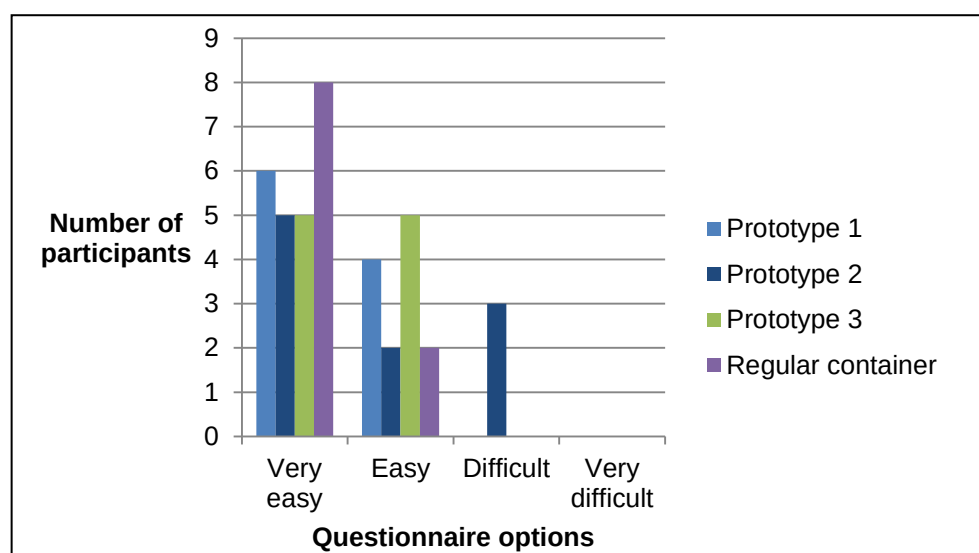


Figure 8. Responses of HCWs to which container would be easiest to instruct patients on correct usage ($n=10$).

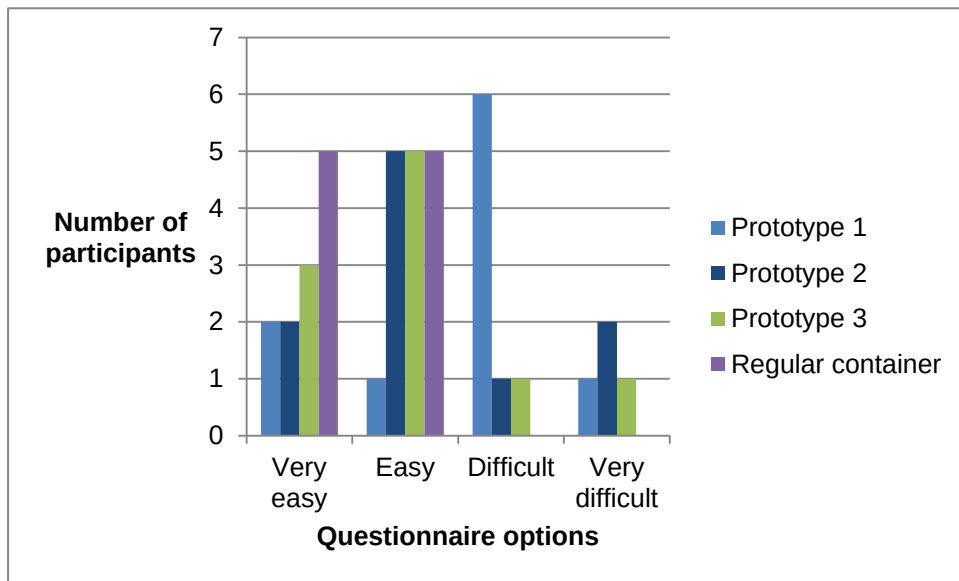


Figure 9. Responses of HCWs to which container would be easiest to transport to the laboratory ($n=10$).

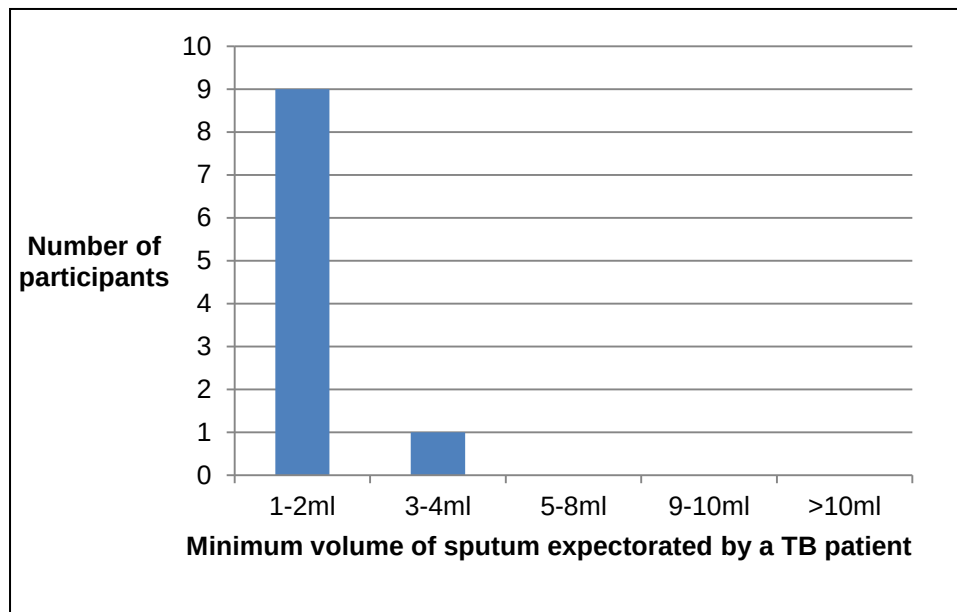


Figure 10. Responses of HCWs to minimum volumes of sputum expectorated by a patient in a single cough attempt ($n=10$).

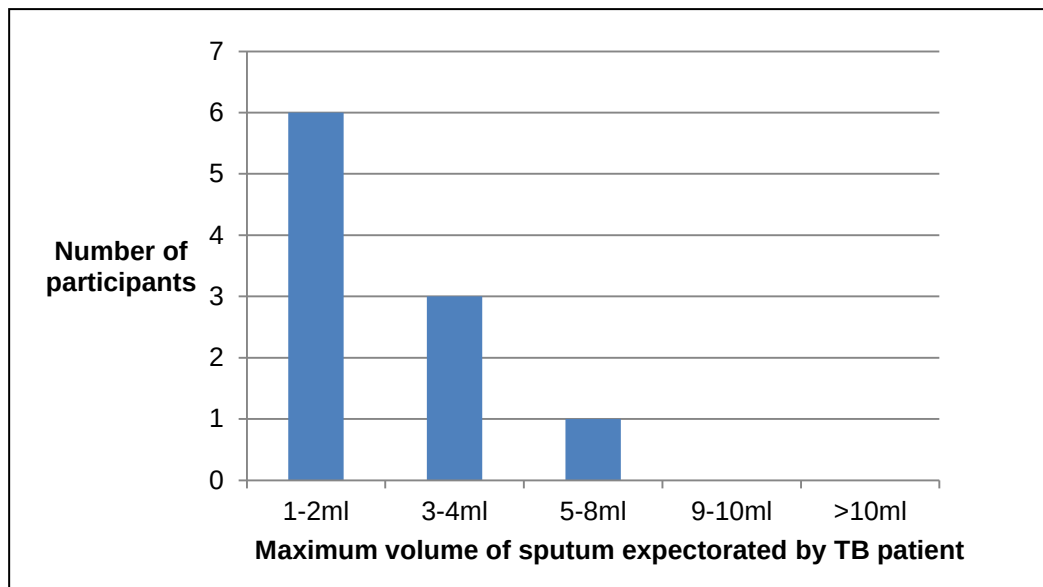


Figure 11. Responses of HCWs to maximum volume of sputum expectorated by a patient in a single cough attempt ($n=10$).

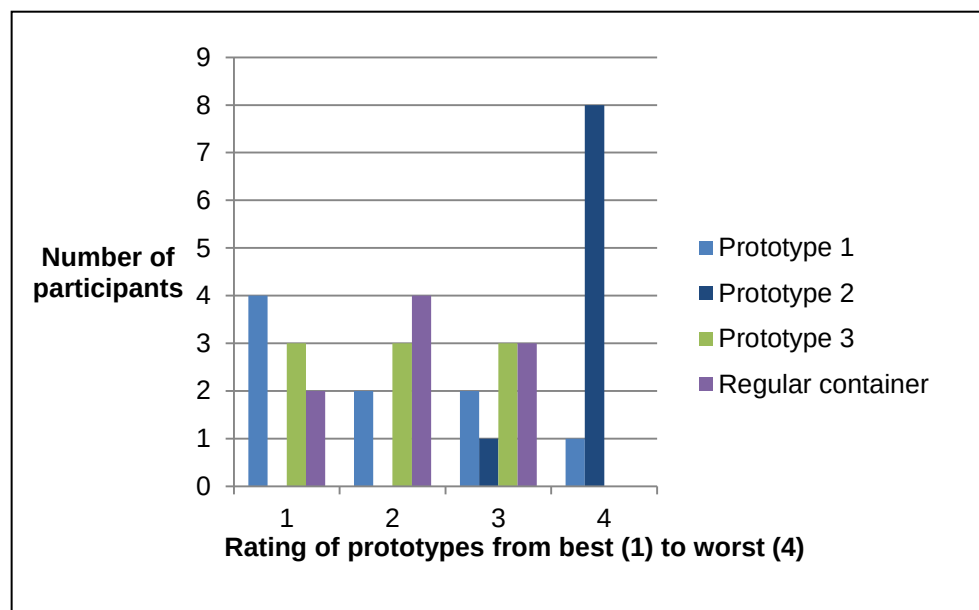


Figure 12. Rating of the prototypes and regular container by the HCWs with 1 (best) and 4 (least favourite) [$n=9$].

4.1.3 Laboratory worker responses

Amongst laboratory workers, the majority (3/5, 60%) felt that the regular container was the easiest to sort (Figure 13). Most of them thought it would be easy to label each of the containers with Prototype 2 being the least favoured. There was not much difference in their opinion on processing with the different containers (Figure 14). Prototype 3 and the regular container were the easiest to use with current laboratory equipment, and the regular container was the easiest to store in the laboratory. The technologists rated Prototype 3 as the best (3/5, 60%) with the regular container following shortly behind (2/5). Prototype 3 and the regular container were the most highly rated choices (Figure 15). The laboratory workers suggested making the containers smaller, marking the containers with volume measurements, and altering the container shape to support batch processing of specimens.

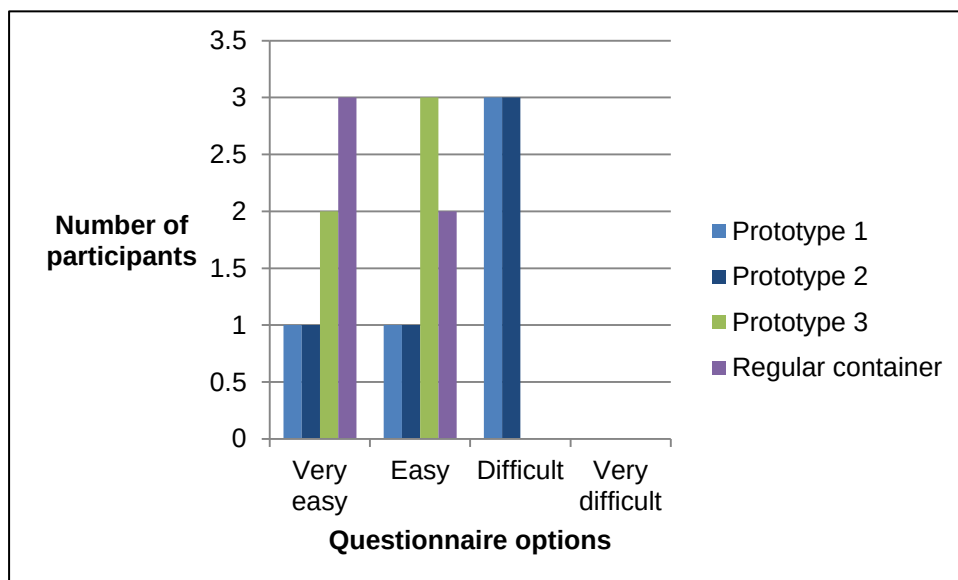


Figure 13. Responses of laboratory workers on the ease of sorting the different prototypes on receipt at the laboratory (n=5).

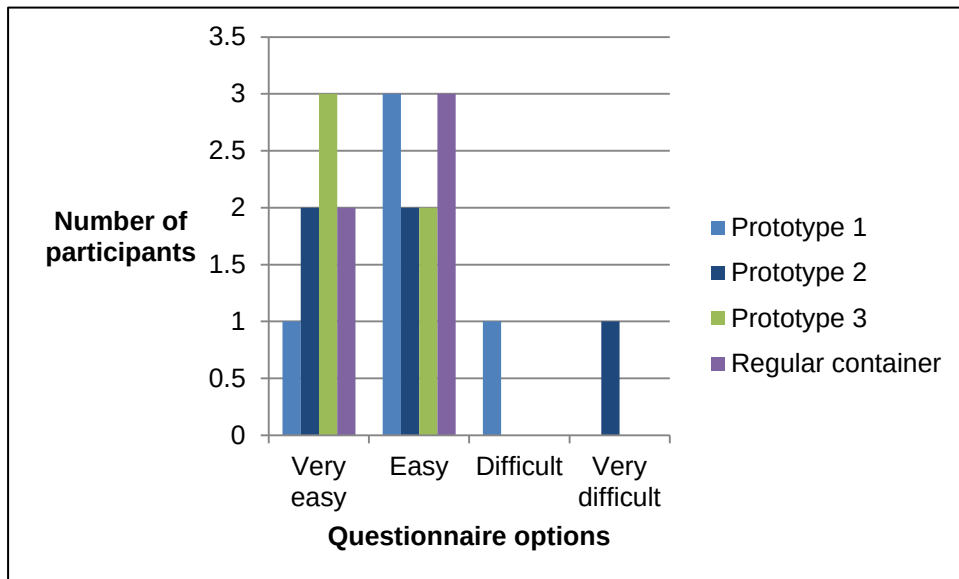


Figure 14. Responses of laboratory workers on the ease of processing sputum specimens in the different prototypes ($n=5$).

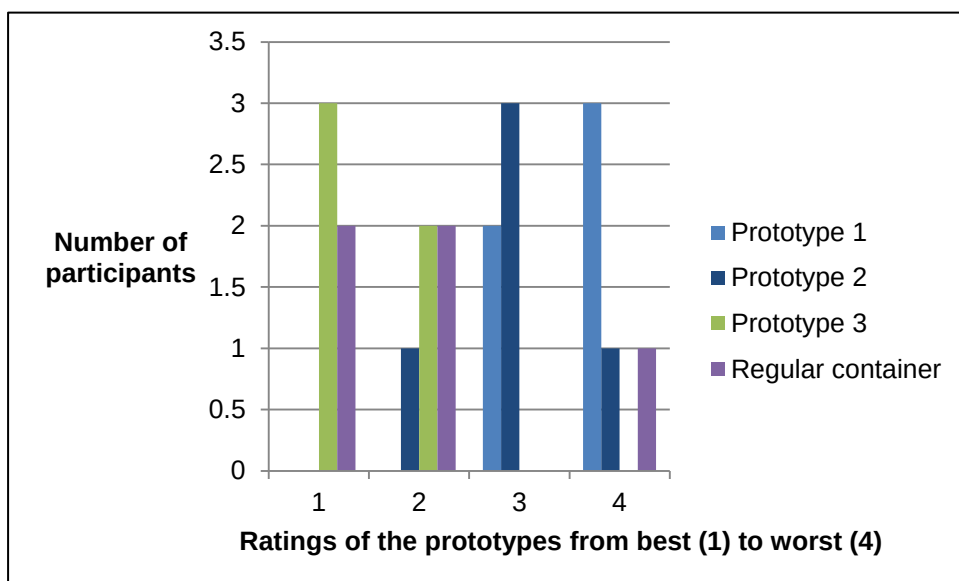


Figure 15. Responses of laboratory workers to rating of each of the prototypes with 1 (best) and 4 (worst) [$n=5$].

Following the results of these questionnaires, Prototype 3 (which was closest to the conventional container) was selected as the final prototype (Figure 16) for further modification and development. Several of the suggestions and comments made by the participants were incorporated into refining the design for the final prototype. These included making the prototypes smaller, marking the containers with volume measurements and altering the base to allow easier processing, storage and transport of the new sputum collection device. Volume indicators on the containers gave visual indicators to patients as to the volume of sputum that needed to be expectorated as well as assisting laboratory workers as to the volume of liquefaction solution that needed to be added to the specimen in a 2:1 ratio. Screw-on lids were used to minimise the risk of leakage of specimens, and a port was added to the lid allowing insertion of liquefaction solution whilst limiting direct exposure to potentially infectious sputum. We removed the liquefaction solution chamber as there were concerns about exposure to potentially toxic reagents should containers be given to patients to take home to collect sputum specimens. The lysis solution would be added by a technician or technologist via the port on the lid on receipt in the laboratory/microscopy centre. This also removed concerns about expired liquefaction solution being added to sputum if the liquefaction solution was incorporated into a chamber in the sputum cup, since unused sputum cups may remain at clinics for extended periods of time.

The new changes were not envisaged to alter patient instruction on sputum collection, or patient expectoration into the cup as the new prototype closely resembled the current container utilised.

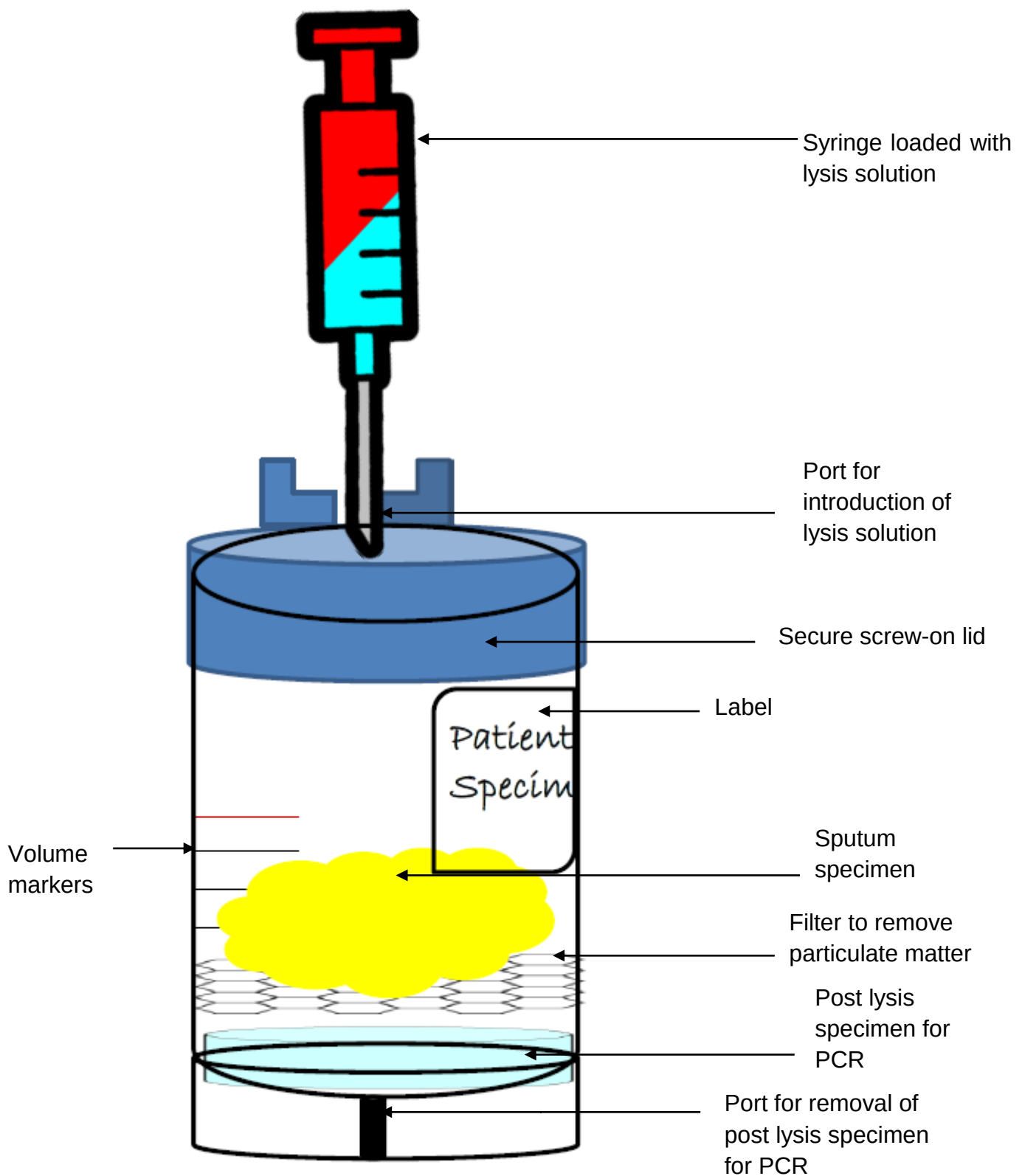


Figure 16. Sketch of proposed new improved prototype conceptualised by student.

The filter was incorporated into the design to remove large particulate material that may be present in sputum and that may potentially interfere with PCR testing. The syringe would be preloaded with sputum liquefaction solution which would be added to the device via the port, limiting exposure of the technician or HCW to potentially infectious sputum.

Our collaborators at CIGHT then developed three prototypes based on the sketch above (Figure 17).

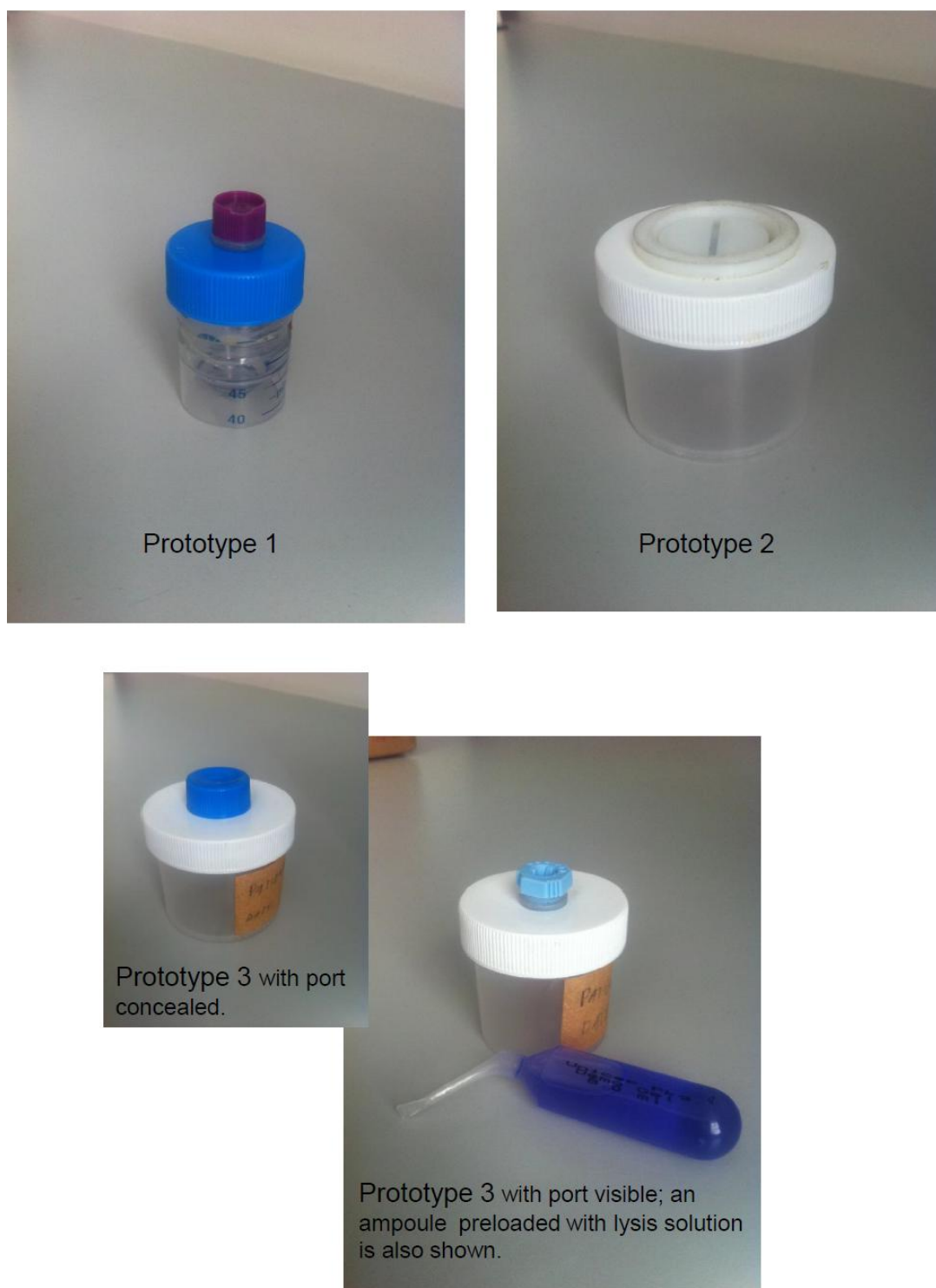


Figure 17. Prototypes developed from revised concept prototype.

Prototype 1 was a plastic container with an inner funnel-shaped collection cup and a lid with port (Figure 17). The second prototype was similar to the

routine container, but the lid concealing the port had to be twisted off with a coin that fitted into the slot on the lid (Figure 17). Prototype 3 was a routine container with a lid with a port without an inner funnelled chamber (Figure 17). Liquefaction solution was injected into the container via the preloaded ampoules (Figure 17). The filter that was initially proposed was not included in the final prototypes produced due to the complexity of incorporating the filter into mass production as well as the increased cost of this manufacturing step.

We informally questioned the laboratory technologists at Groote Schuur Hospital Medical Microbiology NHLS Laboratory, and HCWs at Town 2 clinic, Khayelitsha, on which prototype they preferred. The majority preferred Prototype 1. They felt that it would take too long to remove the lid on Prototype 2 and that Prototype 3 lacked the inner funnel with volume measurements which was preferred. Prototype 1 was then revised and developed further for larger scale production (see Figure 18).

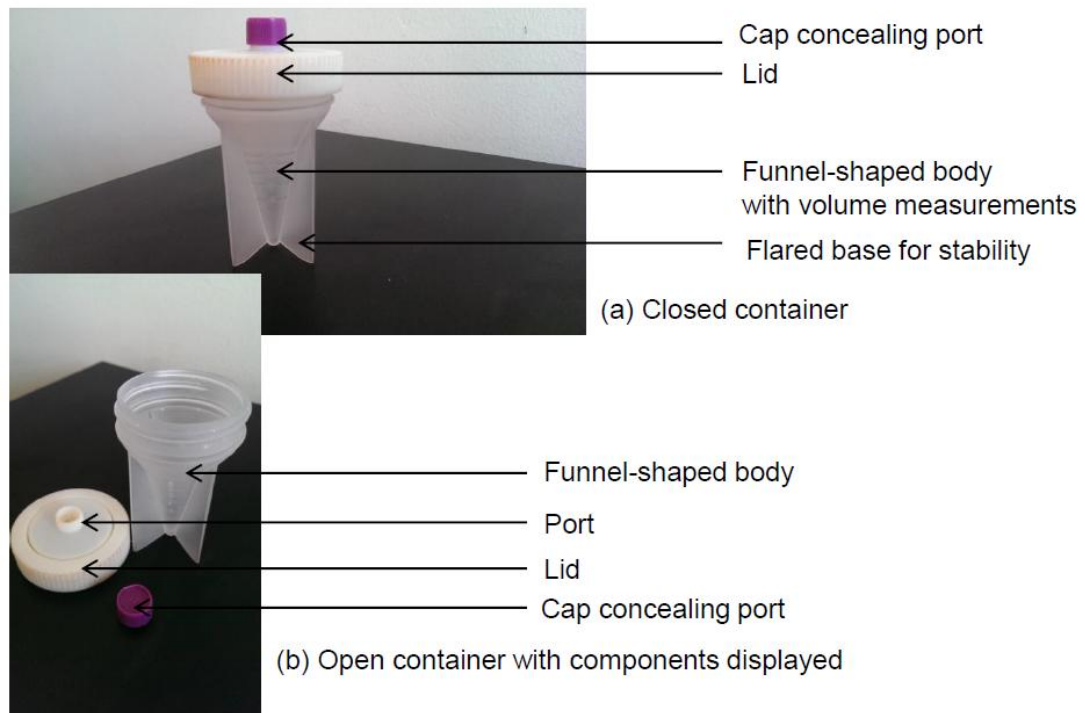


Figure 18. Final prototype. The final prototype measured approximately 43mm x 65mm (outside diameter without lid), and 48mm x 77mm (outside diameter with lid); maximum volume 15mL.

4.2 Second Phase Study

We conducted a limited user acceptability and design validation study. Design validation ensures that devices conform to user needs and includes testing production units under actual use conditions.

4.2.1 Patient participant data

Ninety-three participants suspected of having TB were recruited. All participants (100%) reported that the new container was easy to use. Twenty-three (24.7%) participants specifically commented that they found the new container easier to hold than the regular container, whilst 17 (18.3%)

specifically commented that the new container was much easier to open and close (Figure 19). Eighteen (19.4%) participants specifically commented that it was easier to expectorate into the new container (Figure 19). All specimens fitted easily into the plastic transport sleeves. Of note, no leaking specimens were received at the laboratory and all the lids were closed correctly. No container defects were detected on inspection of the containers on receipt at the laboratory. The volumes of sputum expectorated ranged from 0 mL to 5 mL (mean 1.44 mL; mode 1 mL) (see Figure 20). Five participants were not able to expectorate sputum at the time of testing. Of the participants who were able to provide a sputum specimen, 10 patients produced thick, mucoid sputum; 22 produced thin, mucoid sputum; and 56 produced watery sputum.

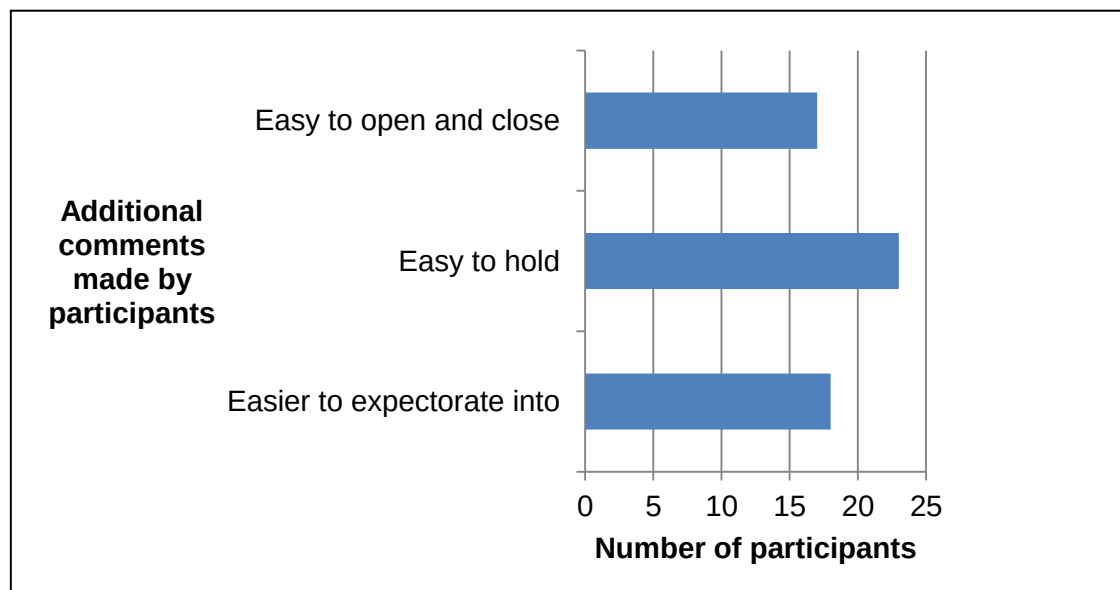


Figure 19. Additional specific comments made by participants regarding the new container.

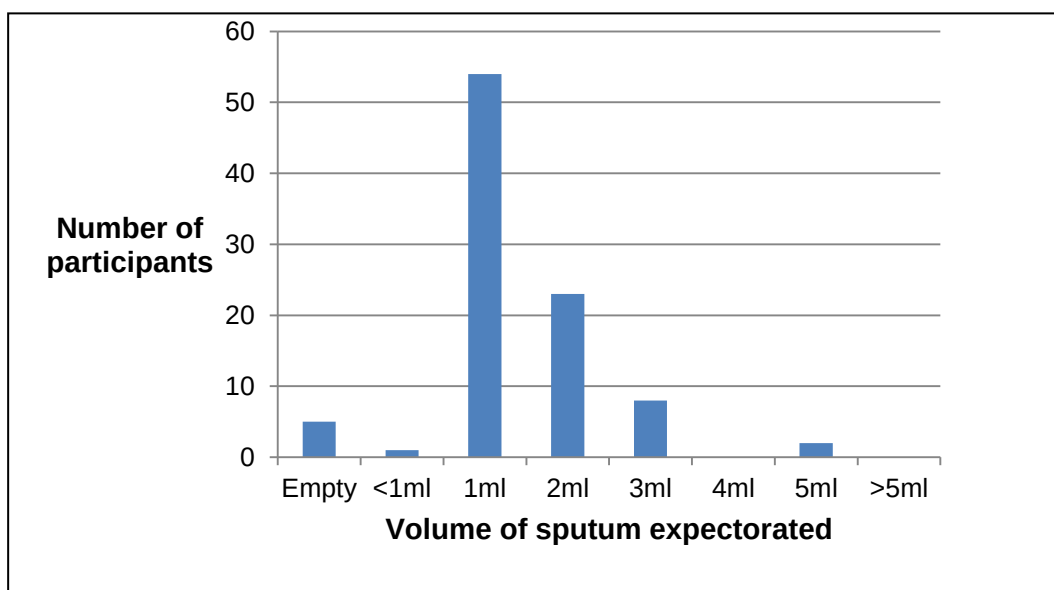


Figure 20. Volumes of sputum expectorated by participants.

A limitation of the new container was the reduction of the maximum volume of sputum that could be deposited into the container due to the inner funnelled chamber design. Limiting the volume of specimen tested could potentially result in a decrease of the sensitivity of the downstream PCR. However, the average volume of 1-2 mL sputum usually expectorated by patients would be easily accommodated in the new prototype. Increasing the size of the container would make it more difficult to transfer and process the specimen within an automated device.

The findings of the functional testing and user acceptability studies are promising. Further studies with functional laboratory testing, product performance qualification and integration into the final device are awaited. These studies are required for the following reasons:

1. To ensure that there is a high probability that all manufactured units will “meet specifications and have uniform quality”. This will minimise scrap or rework resulting in increased output.⁽⁴⁾

2. To ensure “consistent conformance to specifications”: resulting in fewer complaints from patients, HCWs and laboratory staff.⁽⁴⁾
3. The validation data could be used to support further improvements in the sputum containers or in the development of the automated PCR device.⁽⁴⁾

5. DISCUSSION

Tuberculosis is one of the commonest occupationally acquired infections, particularly in third world countries. A Malawian study⁽⁵⁾ conducted in 1996 revealed that the relative risk of TB in HCWs compared to the general population was 11.9 (95% CI [9.8, 14.4]) with a mortality rate of 24%. A study⁽⁶⁾ conducted in Limpopo province, South Africa, between January 2006 to December 2009, revealed that TB accounted for 83.9% ($n=47$) of cases of occupational infection. Alarming, a retrospective study⁽⁷⁾ conducted in Kwa-Zulu Natal (KZN), South Africa, from January 1999 to June 2004 found that the mean incidence of TB to be highest among paramedical staff (including laboratory technicians, radiographers and physiotherapists) when compared to other health professionals (doctors and nurses). These authors concluded that the exposure of laboratory personnel to potentially infectious material whilst preparing a smear put them at higher risk of developing TB.⁽⁷⁾ A more recent study⁽⁸⁾ in KZN found that HCWs had a 2-fold higher risk of developing drug-resistant TB than the general population.

Most laboratory-acquired infections usually occur from activities that generate infectious aerosols that contain *M. tuberculosis*. Smear microscopy is regarded as a low-risk activity as it is believed that very few aerosols will be generated from the manipulation of viscous sputum. The bacillary load ranges from 0 (smear-negative) to 10^3 - 10^4 colony forming units per millilitre (CFU/mL) in a sputum specimen with a scanty smear grading and up to 10^6

CFU/mL in a sample with a 3+grading.⁽⁹⁾ A positive culture contains $>10^8$ CFU/mL.⁽⁹⁾ Thus it is considered that manipulating a sputum specimen is of a lower risk than culture material. The Expert Group of the World Health Organisation (WHO) endorses the preparation of smear microscopy on open benches provided that adequate airflow can be assured.⁽¹⁰⁾ The results of a retrospective Korean study⁽¹¹⁾ which showed that the relative risk of becoming infected with TB for technicians performing direct acid-fast bacilli (AFB) smear microscopy compared with the general population was 1.4 (95% CI [0.2, 10.0]), whilst the risk was 21.5 (95% CI [4.5, 102.5]) for technicians performing drug susceptibility testing (DST) was used to support the WHO recommendation. Although this may be true, the cumulative risk of exposure in high burden countries such as Swaziland (where the incidence rate in 2012 was 1,350/100,000 population⁽¹²⁾) and South Africa (where the incidence rate in 2012 was 1,000/100,000 population⁽¹²⁾) versus Korea (incidence rate of 409/100,000 population⁽¹²⁾) may put laboratory workers at much higher risk. In addition, the infective dose of *M. tuberculosis* is low [Infective Dose₅₀ (ID₅₀) <10 bacilli].⁽¹³⁾ The WHO recommendation⁽¹⁰⁾ is also at odds with the recommendations of the Centers for Disease Control (CDC) which recommend preparation of smears in a biosafety cabinet due to the potential generation of aerosols, droplets and splashes.⁽¹⁴⁾

A study investigating the bioaerosol infection risk of the Xpert®MTB/RIF assay (Cepheid, Sunnyvale, CA) using sputum spiked with *Mycobacterium bovis* BCG at 5×10^8 found that 16 ± 2.3 CFU/m³ (colony forming units per cubic meter) via the N-6 viable Anderson cascade impactor and 325 ± 112.5 CFU/m³ via Biosampler were detected during preparation of smear microscopy.⁽¹⁵⁾ When Xpert®SR solution (sample treatment reagent) was added to the specimen and the cartridge was loaded without incubation (i.e. incorrect usage of SR solution), viable aerosol generation was still detected (6 ± 5.5 CFU/m³) via the Anderson impactor and 67 ± 115.4 CFU/m³ via Biosampler.⁽¹⁵⁾ Therefore, initial manipulation of the sputum specimen for

the Xpert®MTB/RIF assay or other nucleic acid amplification tests (NAATs) prior to incubation with SR solution [which effectively sterilises *M. tuberculosis*⁽¹⁵⁾] or incorrect use of the SR solution, may pose an infectious aerosol risk to laboratory workers.

Whilst relatively low risk, the procedure of sputum smear microscopy or manipulation of specimen for NAATs has the potential to generate infectious aerosols. For this reason, it is recommended that AFB microscopy centres ensure adequate ventilation to minimise the risk of occupationally acquired infection. This, however, may not always be feasible in areas of limited airflow or where the use of open windows is restricted within a building. In addition, individuals working in a laboratory differ in their susceptibility to develop TB, and HIV-infected laboratory workers may be at higher risk of lab acquisition of TB. Exposure to low levels of aerosols may result in clinical disease in these individuals. To overcome this problem, the CDC and the Global Laboratory Initiative of the STOP TB Partnership in association with the International Union Against Tuberculosis and Lung Disease and the Association of Public Health Laboratories have validated a ventilated workstation to ensure a safe laboratory work environment for use during sputum smear microscopy as well as manipulation of specimens for Xpert® testing.⁽⁹⁾ However microbiological biosafety cabinets are expensive equipment, require continual monitoring and yearly maintenance checks by qualified personnel. This equipment is unlikely to be available in rural facilities in low-income settings.

The lack of biosafety for staff performing TB tests was clearly evident in a survey conducted to assess the feasibility of rolling out POC NAATs in 22 high burden countries.⁽¹⁶⁾ N95 respirators were not available in 12 countries and inconsistently available in seven countries. Safety hoods were available

in only three countries, and inconsistently available in five countries (Figure 21).

	Country	Environment		Infrastructure			Available equipment							
		Temp.	Humidity	Power	Water	Security	Gloves	N95	Pipettes	Refrigerator	Incubator	Centrifuge	Water bath	Hood
Non-BRICS	Congo													
	Zimbabwe													
	Mozambique													
	Ethiopia													
	Afghanistan													
	Myanmar													
	Uganda													
	Tanzania													
	Kenya													
	Bangladesh													
	Cambodia													
	Nigeria													
	Pakistan													
	Vietnam													
	Philippines													
Indonesia														
Thailand														
BRICS	India													
	China													
	South Africa													
	Brazil													
	Russia													

	Country	Skills			Communication			QA established	Stockouts	Waste established	Current testing	
		Pipetting	PCR tests	Computer	Landline	Mobile	Internet				ZN	FM
Non-BRICS	Congo											
	Zimbabwe											
	Mozambique											
	Ethiopia											
	Afghanistan											
	Myanmar											
	Uganda											
	Tanzania											
	Kenya											
	Bangladesh											
	Cambodia											
	Nigeria											
	Pakistan											
	Vietnam											
	Philippines											
Indonesia												
Thailand												
BRICS	India											
	China											
	South Africa											
	Brazil											
	Russia											

Yes/present

Maybe

No/not present

Unsure/question not answered

Figure 21. Characteristics of microscopy centres in 22 high burden countries.⁽¹⁶⁾ Reproduced with permission of the European Respiratory Society ©. *European Respiratory Journal* Aug 2013, 42 (2) 544-547; DOI: 10.1183/09031936.00081113

The authors concluded that there are many barriers to POC testing, and test developers need to align their ideal target product profiles with the realistic conditions in which most national TB programmes operate, especially if the goal is same-day diagnosis and treatment at the POC.⁽¹⁶⁾

6. CONCLUSION

Our redesigned sputum collection device offers a cheap, effective solution to biosafety concerns with minimal impact on patient acceptability and clinical care. It is envisaged that these new containers could easily be manufactured locally in low- and middle-income countries reducing the cost of production. They can be easily adapted for use with various other NAATs. An additional advantage of these containers is that the prototype can easily be customised to collect other potentially infectious body fluids for molecular testing whilst limiting exposure of healthcare workers and laboratory workers to these fluids.

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APPENDIX

Appendix 1. Phase 1 study-Questionnaire for participants suspected of having TB.

Appendix 2. Phase 1 study-Questionnaire for healthcare workers.

Appendix 3. Phase 1 study- Questionnaire laboratory workers.

Appendix 4. Phase 2 study- Instructions given to patients.

Appendix 5. TB poster used to illustrate sputum collection for the participants.

Appendix 6. Phase 2 study-Informed consent form.

Appendix 7. Phase 2 study- Clinical research form.

Appendix 1. Phase I study-Questionnaire for participants suspected of having TB.

Description of the three prototypes

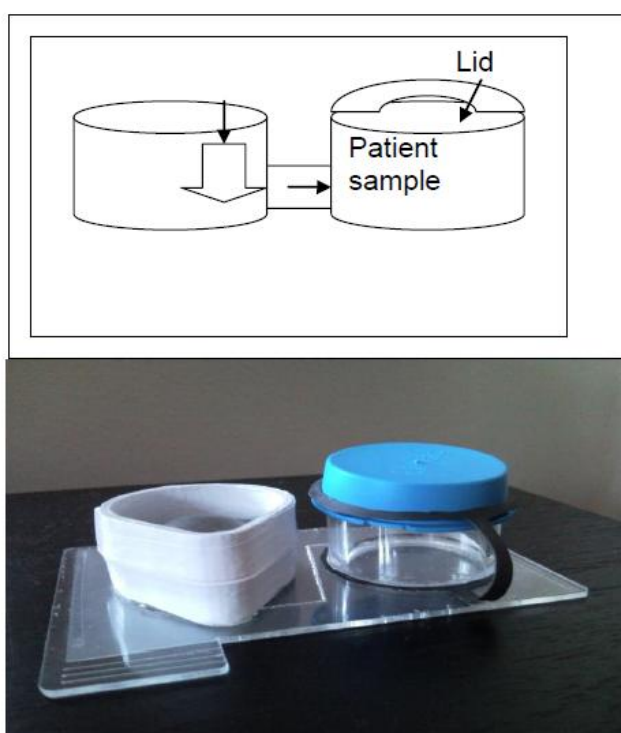
The three sputum containers have been developed to allow safe and efficient collection of sputum and killing of the TB germ. This system will allow testing for TB in a closed system with no danger to healthcare workers.

PROTOTYPE 1:

You will be asked to cough up some sputum into the plastic bag through the funnel at the top. The blue lid is then closed, and the specimen is taken back to the clinic.

PROTOTYPE 2:

You will be asked to cough up some sputum into in the container on the right. The blue lid is then closed, and the specimen is taken back to the clinic.



PROTOTYPE 3:

You will be asked to cough up some sputum into the sputum pot. The lid is closed tightly and taken back to the clinic.



QUESTIONNAIRE: EVALUATION OF INTEGRATED SPUTUM COLLECTION DEVICES

INVESTIGATORS: *Prof Mark Nicol, Dr Mischka Moodley, Prof David Kelso*
University of Cape Town, South Africa/ Northwestern University, USA.

We would like you to help us decide which of these containers for collecting sputum for TB testing is best, and whether there are any problems associated with these new containers. We would appreciate your help by answering some questions about the new containers. Your opinion is important as it will help us decide which of the containers are best for further development. The questionnaire should not take more than 20 minutes to fill in. The answers you give will be confidential and will not be discussed with anyone, other than the people conducting this survey. You may, however, choose not to take part. This will not affect your treatment at this clinic.

Please put a cross in the appropriate box. For example, ☒

1. Please rate each type of sputum container on how easy you think it would be to cough into.

		Very easy	Easy	Difficult	Very difficult
Prototype 1		☐	☐	☐	☐
Prototype 2		☐	☐	☐	☐
Prototype 3		☐	☐	☐	☐
Regular container		☐	☐	☐	☐

2. Please rate each of the containers on how easy you think it would be to take home to get a sputum specimen, for example, put it in your handbag or pocket.

		Very easy	Easy	Difficult	Very difficult
Prototype 1		☐	☐	☐	☐
Prototype 2		☐	☐	☐	☐
Prototype 3		☐	☐	☐	☐
Regular container		☐	☐	☐	☐

3. **Please rate how easy you think it will be to open and close each container.**

		Very easy	Easy	Difficult	Very difficult
Prototype 1		☐	☐	☐	☐
Prototype 2		☐	☐	☐	☐
Prototype 3		☐	☐	☐	☐
Regular container		☐	☐	☐	☐

4. **How would you rank the containers in order of your preference with 1(the one you like the most) and 4(the one you like the least)? Please indicate the number in the box.**

Prototype 1		☐
Prototype 2		☐
Prototype 3		☐
Regular container		☐

5. **Would you feel uncomfortable using the new containers?**

Yes	☐	No	☐

If yes, which one.

Prototype 1		☐	Prototype 2		☐
Prototype 3		☐	Regular container		☐

If yes, please tell us why.

Appendix 2. Phase I study-Questionnaire for healthcare workers.

Confidential

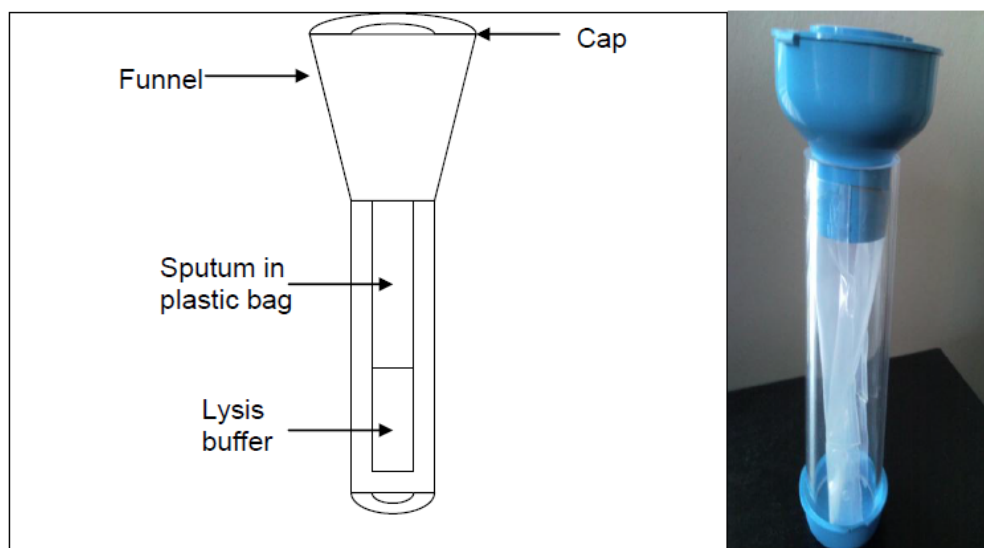
Page 1 Healthcare worker

Description of the three prototypes

The three sputum containers have been developed to facilitate safe and efficient collection of sputum and killing of Mycobacterium tuberculosis. This system will allow DNA testing in an entirely closed system with no risk to laboratory or clinic staff.

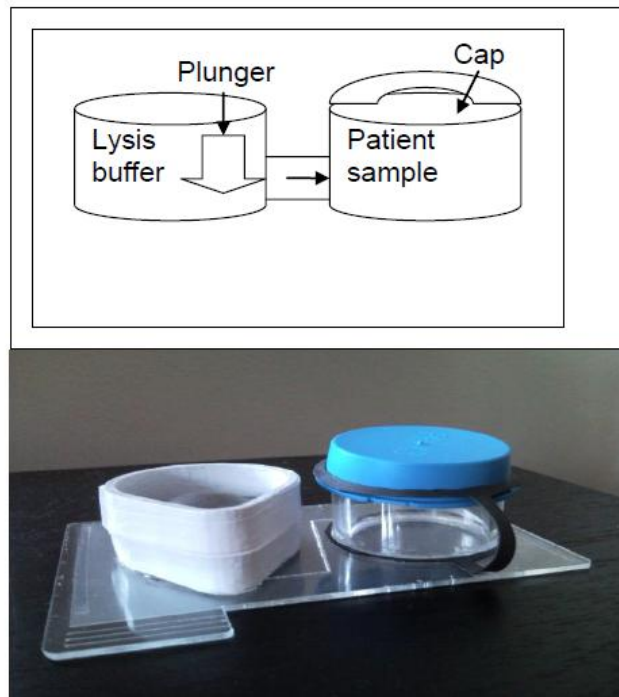
CONTAINER 1:

The patient will be instructed to deposit a sputum specimen into the plastic bag via the funnel at the top. The blue lid is closed tightly, and the specimen is transported to the laboratory



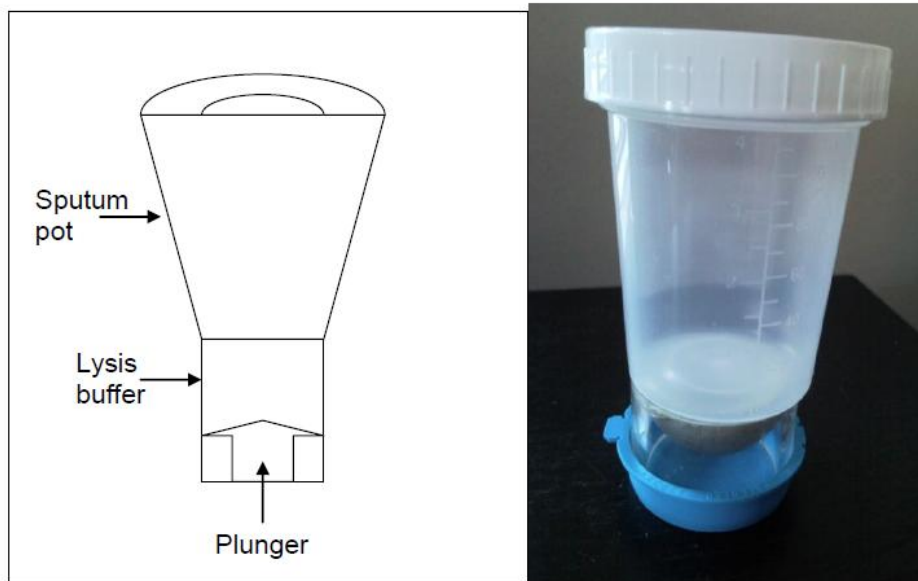
CONTAINER 2:

The patient is instructed to produce a sputum sample and deposit it in the container on the right. The blue cap is closed tightly, and the specimen is transported to the laboratory.



CONTAINER 3:

The patient is instructed to produce a sputum sample. The specimen is deposited into the sputum pot. The white lid is closed tightly, and transported to the laboratory.



QUESTIONNAIRE: EVALUATION OF INTEGRATED SPUTUM COLLECTION DEVICES

INVESTIGATORS: *Prof Mark Nicol, Dr Mischka Moodley, Prof David Kelso*

We would like you to help us decide which of these containers for collecting sputum for TB testing is best, and whether there are any problems associated with these new containers. We would appreciate your help by answering some questions about the new containers. Your opinion is important as it will help us decide which of the containers are best for further development. The questionnaire should not take more than 20 minutes to fill in. The answers you give will be confidential and will not be discussed with anyone, other than the people conducting this survey. You may, however, choose not to take part.

Please put a cross in the appropriate box. For example,

X

1. Please rate how easy it would be to give the patient instructions about sputum collection using each of the containers.

		Very easy	Easy	Difficult	Very difficult
Prototype 1		☐	☐	☐	☐
Prototype 2		☐	☐	☐	☐
Prototype 3		☐	☐	☐	☐
Regular container		☐	☐	☐	☐

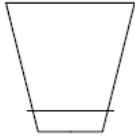
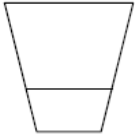
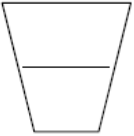
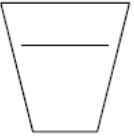
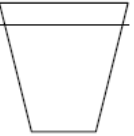
2. Please rate how easy it would be to label each of the specimen containers with the patient details and folder number.

		Very easy	Easy	Difficult	Very difficult
Prototype 1		☐	☐	☐	☐
Prototype 2		☐	☐	☐	☐
Prototype 3		☐	☐	☐	☐
Regular container		☐	☐	☐	☐

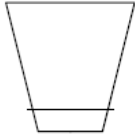
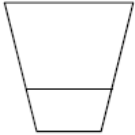
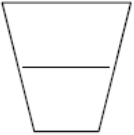
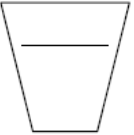
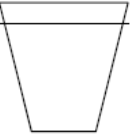
3. **Please rate how easy it will be to transport each of the specimen containers to the laboratory.**

		Very easy	Easy	Difficult	Very difficult
Prototype 1		☐	☐	☐	☐
Prototype 2		☐	☐	☐	☐
Prototype 3		☐	☐	☐	☐
Regular container		☐	☐	☐	☐

4. **In your experience, what is the minimum volume of sputum you think a patient can cough up in a single cough?**

				
☐	☐	☐	☐	☐

5. **In your experience, what is the maximum volume of sputum you think a patient can cough up in a single cough?**

				
☐	☐	☐	☐	☐

6. How would you rank the containers in order of your preference with 1-best and 4-worst? Please indicate the number in the box.

Prototype 1



Prototype 2



Prototype 3



Regular container



7. Please suggest any changes that you can think of which would improve sputum container design.

THANK YOU FOR YOUR ASSISTANCE.

Appendix 3. Phase I study- Questionnaire laboratory workers.

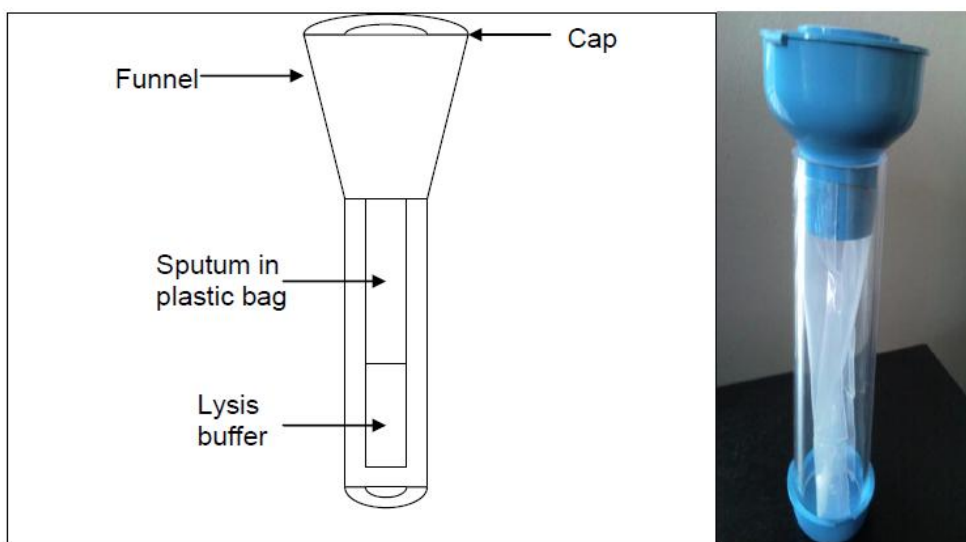
Confidential Laboratory worker

Description of the three prototypes

*The three sputum containers have been developed to facilitate safe and efficient collection of sputum and lysis of *Mycobacterium tuberculosis*. This system will permit DNA extraction in an entirely closed system with no risk to laboratory staff.*

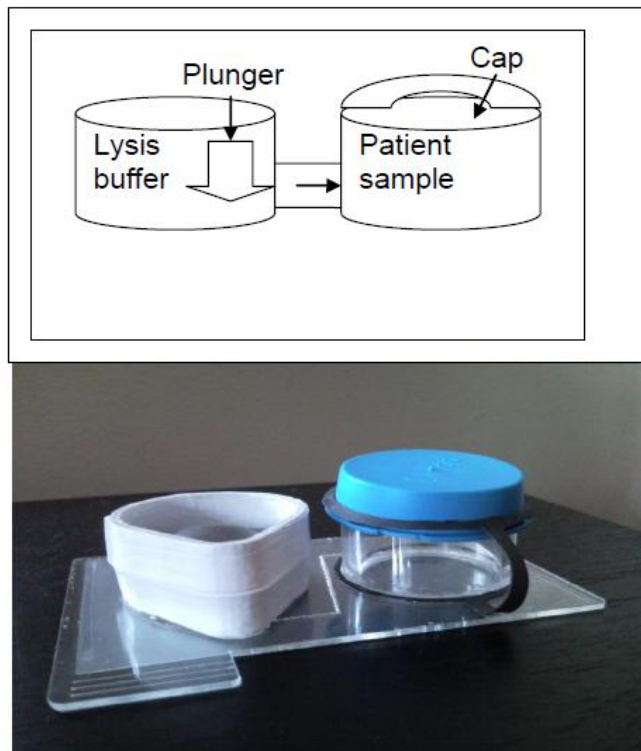
PROTOTYPE 1:

The specimen is deposited into the plastic bag via the funnel at the top. The blue lid is closed and the specimen is transported to the laboratory. At the laboratory, the cap at the bottom is removed and the lysis buffer blister chamber is depressed, allowing the lysis buffer to interact with the sputum sample. The tube is inverted several times.



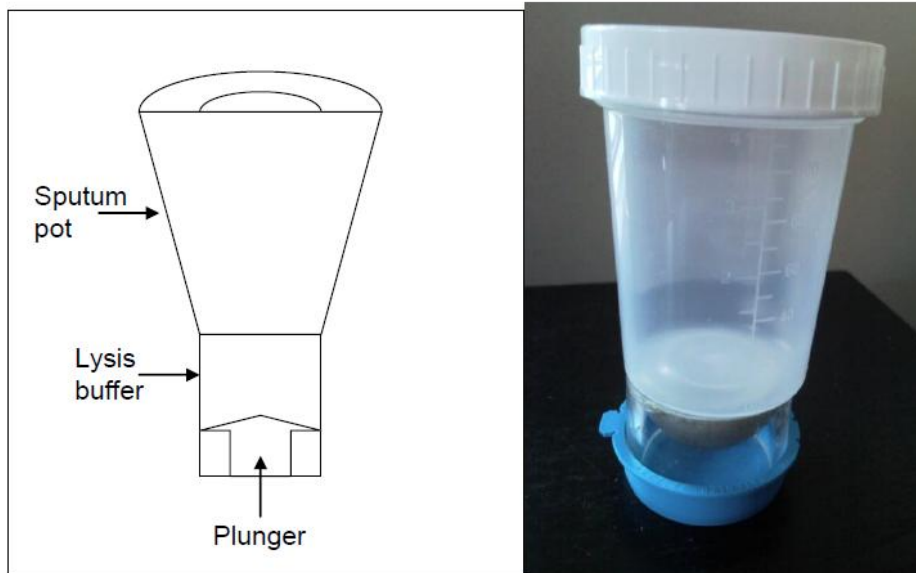
PROTOTYPE 2:

The patient sputum sample is deposited in the container on the right. The specimen is transported to the laboratory. At the laboratory, the lysis buffer chamber is depressed; a plunger then forces the lysis buffer into the patient sample. The container is inverted several times to allow mixing of the specimen and lysis buffer.



PROTOTYPE 3:

The specimen is deposited into the sputum pot and transported to the laboratory. At the laboratory, the plunger at the bottom of the container is depressed, releasing the lysis buffer into the sputum. The container is inverted several times to allow mixing of the sample with the lysis buffer.



QUESTIONNAIRE: EVALUATION OF INTEGRATED SPUTUM COLLECTION DEVICES

INVESTIGATORS: *Prof. Mark Nicol, Dr. Mischka Moodley, Prof. David Kelso*
University of Cape Town, South Africa/ Northwestern University, USA.

We would like you to help us decide which of these containers for collecting sputum for TB diagnosis is best, and whether there are any problems associated with these new containers. We would appreciate your help by answering some questions about the new containers. Your opinion is important as it will help us decide which of the prototypes are best for further development. The questionnaire should not take more than 20 minutes to fill in. The answers you give will be confidential and will not be discussed with anyone, other than the people conducting this survey. You may, however, choose not to take part.

Please put a cross in the appropriate box. For example,

X

1. Please rate how easy it will be to sort each type of container on receipt at the laboratory.

		Very easy	Easy	Difficult	Very difficult
Prototype 1		☐	☐	☐	☐
Prototype 2		☐	☐	☐	☐
Prototype 3		☐	☐	☐	☐
Regular container		☐	☐	☐	☐

2. Please rate how easy it will be to label each type of container.

		Very easy	Easy	Difficult	Very difficult
Prototype 1		☐	☐	☐	☐
Prototype 2		☐	☐	☐	☐
Prototype 3		☐	☐	☐	☐
Regular container		☐	☐	☐	☐

3. **Please rate how easy it will be to process sputum samples received in each of the containers.**

		Very easy	Easy	Difficult	Very difficult
Prototype 1		☐	☐	☐	☐
Prototype 2		☐	☐	☐	☐
Prototype 3		☐	☐	☐	☐
Regular container		☐	☐	☐	☐

4. **Please rate how easy it will be to use each type of container with the current equipment in the laboratory, for example, pipettes.**

		Very easy	Easy	Difficult	Very difficult
Prototype 1		☐	☐	☐	☐
Prototype 2		☐	☐	☐	☐
Prototype 3		☐	☐	☐	☐
Regular container		☐	☐	☐	☐

5. Please rate how easy it would be to store each of the containers in the laboratory, for example, in the refrigerator for after-hours specimens.

		Very easy	Easy	Difficult	Very difficult
Prototype 1		☐	☐	☐	☐
Prototype 2		☐	☐	☐	☐
Prototype 3		☐	☐	☐	☐
Regular container		☐	☐	☐	☐

6. Please rank the containers in order of your preference from 1 (the container you like the most) to 4 (the container you like the least). Please indicate the number in the box.

Prototype 1		☐
Prototype 2		☐
Prototype 3		☐
Regular container		☐

7. Please suggest any changes that you can think of which would improve sputum container design.

THANK YOU FOR YOUR ASSISTANCE.

Appendix 4. Phase 2 study- Instructions given to patients.

Patient Instructions Pamphlet

The research worker will give you a special plastic cup for collecting your sputum. Follow these steps carefully.

1. The cup is very clean. Do not open it until you are ready to use it.

2. Rinse your mouth well with water.

3. Go outside the clinic or into the sputum room before collecting the sputum sample. This helps protect other people from TB germs when you cough.

4. Take a very deep breath and hold the air for 5 seconds. Slowly breathe out.

Take another deep breath and cough hard until some sputum comes up into your mouth.

5. Open the container. Place the open container close to your mouth to collect the specimen. Spit the sputum into the plastic cup. Avoid touching the inside of the container and lid.

6. Keep doing this until the sputum reaches the 2 ml line (red line) on the plastic cup. This is about 1/2 teaspoon of sputum.

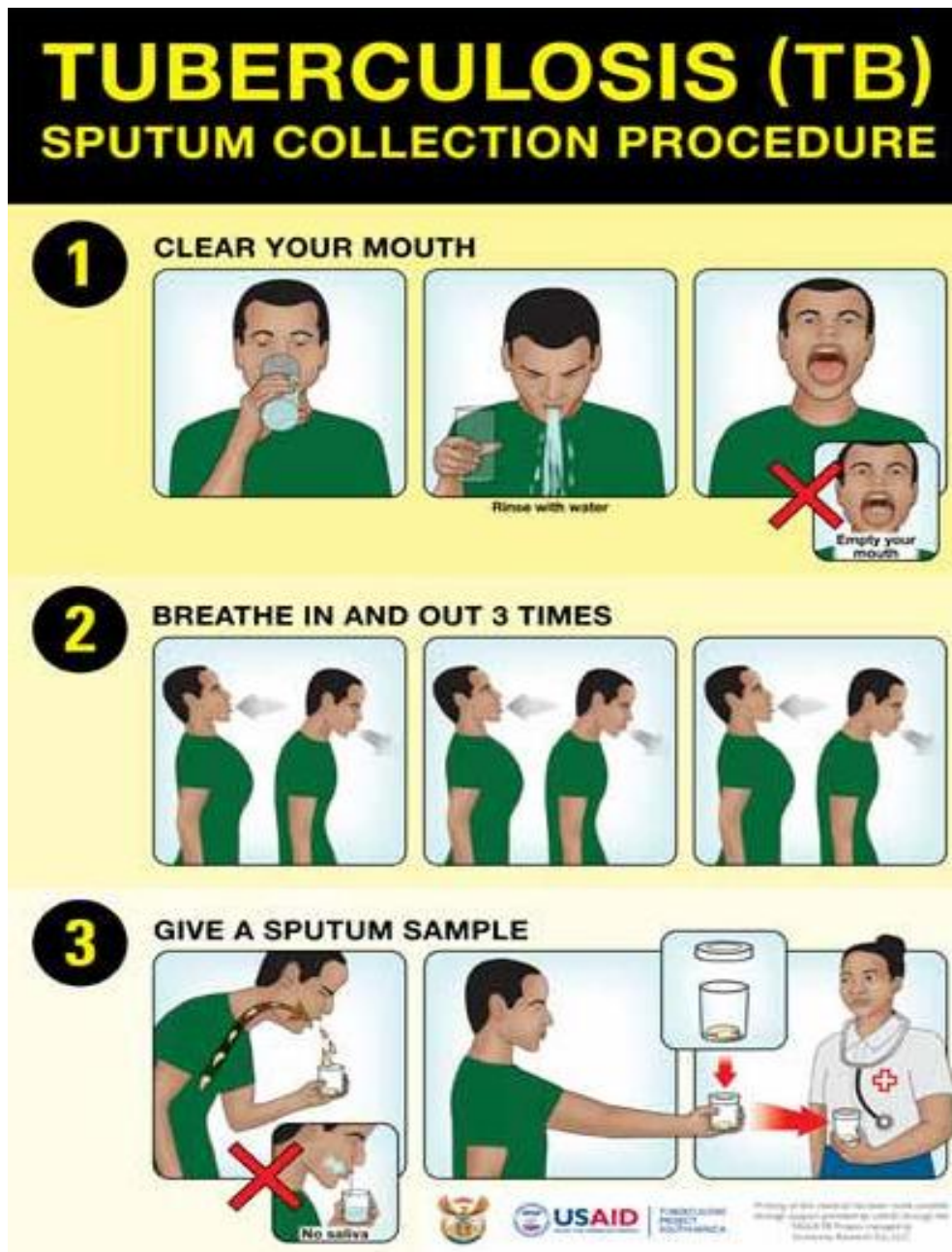
7. Screw the cap on the cup tightly so it doesn't leak.

8. Wipe the outside of the cup with a paper towel.

9. Put the cup into the plastic bag the nurse gave you.

10. Give the cup to your clinic or nurse.

Appendix 5. TB poster used to illustrate sputum collection for the participants.



Accessed from:

<https://findtbresources.cdc.gov/newsletter/newsletter11dec.html>

Appendix 6. Phase 2 study-Informed consent form (English version).

Sputum collection device Protocol Version 2.0 ICF_Version 2.0_15 March 2012
Principal investigators: Prof Mark Nicol/ Dr Mischka Moodley/ Prof David Kelso
Site of Research: UCT

INFORMED CONSENT FORM: PARTICIPANTS

Study Title: Integrated Microanalytical Extraction-Amplification System for Detection of Tuberculosis in Low Resource Settings : To design and test an integrated sputum collection/processing container

Dear Sir /Madam,

You have been told by your clinic doctor that you may have tuberculosis (TB) and that you will have to have some tests done to try to see if you really have TB or not. These tests are usually done on sputum (phlegm) that you cough up. Because the phlegm contains the TB bacilli, it is infectious, which means that the clinic healthcare workers and laboratory staff can get TB from this phlegm.

Researchers from the University of Cape Town (Professor Mark Nicol from the Division of Medical Microbiology) as well as Northwestern University in the United States have designed a new sputum cup that may help to protect healthcare workers and lab staff from getting infected in this way. We are doing research on this new sputum cup. We want to test how easy it is to cough into, as well as whether the container is strong, does not leak, etc.

We want to ask you if you would be willing to be part of this study on the new sputum cup. To be part of this study, you must be older than 18 years and you must also be able to cough up sputum.

If you agree to be part of the study, we will ask you to cough up a sputum (phlegm) specimen into the new cup. The clinical research worker will explain to you how to cough into the cup. This sputum will not be tested for TB. You will need to provide sputum specimens to the clinic nurse or doctor which will have tests done for TB.

There are no risks to you if you decide to take part in this study. The results of the testing that we do on the sputum cup will not help you in any way – but what we eventually learn when the whole study is finished might help other people in the future. You will be given R50 for the time that you have used taking part in the study.

Throughout the study, your privacy will be maintained. Nobody other than the study doctors and nurses will know that you are taking part in this study. None of the results from this study will be given to your clinic doctors or nurses who are treating you. Even when the study is finished, no person's name will be shown in publications.

The decision to participate is entirely your own. If you decide not to participate in the study, this will not disadvantage your treatment in any way. It is completely up to you if, at any point, you choose to tell us that you do not want to be part of the study anymore. If you do withdraw from the study, at any point, this will not in any way have an effect on the treatment you receive from your clinic doctors or nurses.

This study will be monitored by the Research Ethics Committee of the University of Cape Town. During the study you may contact either the UCT Research Ethics Committee (021 406 6492, or the principal investigator (021 406 6083) if you have further questions. Please remember that these people are however not directly responsible for your medical care, which is the job of your clinic doctors and nurses.

Please ask the study researcher now if you have any questions about the study or about your decision to be involved or not. If you have decided to be part of the study, please fill in your full name, your signature and the date below.

Your signature on this form means that you understand the information given to you in this form, you accept the provisions in the form and that you agree to join the study. You will not give up any legal rights by signing this consent form.

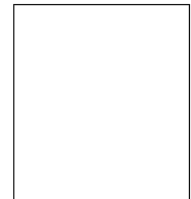
Sputum collection device Protocol Version 2.0 ICF_Version 2.0_15 March 2012
Principal investigators: Prof Mark Nicol/ Dr Mischka Moodley/ Prof David Kelso
Site of Research: UCT

WE WILL GIVE YOU A COPY OF THIS SIGNED AND DATED CONSENT FORM.

I have read the information, or it has been read to me. I have had the opportunity to ask questions about it and my questions have been answered to my satisfaction. I consent voluntarily and understand that I have the right to withdraw my consent without this affecting the current research study or my medical care.

Full name and Signature of Participant

Date



Right
thumbprint

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Full name and Signature of Person Obtaining Consent

Date

Full name and Signature of Witness

Date

NOTE: A COPY OF THE SIGNED, DATED CONSENT FORM MUST BE KEPT BY THE PRINCIPAL INVESTIGATOR; A COPY MUST BE GIVEN TO THE PARTICIPANT; AND, IF APPROPRIATE, A COPY OF THE CONSENT FORM MUST BE PLACED IN THE PARTICIPANT'S MEDICAL RECORD.

Copy provided to participant _____

(Initialed by researcher)

Appendix 7. Phase 2 study-Clinical research form.

Sputum collection device – Prof Nicol/ Dr Moodley/ Prof Kelso
UCT/ Northwestern University

CRF V1.0_15 March2012

CLINICAL RESEARCH FORM

PATIENT INFORMATION:

Patient Initials _____

Date of Birth _____

SCREENING:

1. Older than 18 years	☐ Yes	☐ No
2. Able to give informed consent	☐ Yes	☐ No
3. Willing to provide additional specimen	☐ Yes	☐ No
4. Able to expectorate additional specimen	☐ Yes	☐ No
5. Able to follow instructions	☐ Yes	☐ No

If answered yes to questions 1-5 above, attach signed consent form. Instruct patient on how to collect sputum specimen and provide a sputum container.

Patient evaluation:

1. Was the container easy to use? ☐ Yes ☐ No

If no, what was the reason. _____

2. Any additional comments from the patient. _____

Evaluation of specimen by CRW:

1. Is the specimen received in the plastic bag? ☐ Yes ☐ No

If no, provide reason. _____

2. Any specimen leakage? ☐ Yes ☐ No

If yes, provide description. _____

3. Is the lid attached correctly? ☐ Yes ☐ No

If no, provide description. _____

4. What is the volume of the specimen received? Round off value to nearest whole number.

☐ Empty ☐ < 1 ml ☐ 1 ml ☐ 2ml ☐ 3ml ☐ 4ml ☐ ≥ 5ml

5. What is the quality of the sputum?

☐ Muroid, thick ☐ Muroid, thin ☐ Watery

☐ Other. Please describe. _____

6. Colour of the sputum

☐ Yellow ☐ White ☐ Green ☐ Red/brown

☐ Other. Please describe. _____

7. Any defects in physical structure of container (cracks or leaks). ☐ Yes ☐ No

If yes, please describe. _____

FINAL CONCLUSION

There have been tremendous improvements in diagnostic tests for tuberculosis (TB) over the last few years. Molecular testing and the advancement of technology are revolutionising the approach to diagnosis.

Despite these advances, no test is able to meet all the required specifications in terms of performance (sensitivity and specificity), ease of use, cost, rapidity to result, and robustness. Approximately 3.6 million TB cases are estimated to be missed every year which implies a failure of diagnosis, treatment and/or notification to the public health system.⁽¹⁾ Multisectorial action, which includes the development and implementation of new diagnostics, is required if we are to achieve the Sustainable Development Goal of reducing TB-related mortality by 95% and reducing the incidence of TB by 90% by 2035.⁽²⁾ Early diagnosis and treatment of TB is an important component of the global TB strategy.⁽³⁾ Without new diagnostic tests, it will not be possible to accelerate incidence decline by 17% per year, which is required to achieve the 2035 targets.^(2, 4, 5)

Point-of-care (POC) diagnostics for TB are critical in resource-limited settings, where the largest burden of TB lies. Despite numerous hurdles facing diagnostic test developers and manufacturers, new tests continue to be developed, however none to date meet the required specifications.⁽⁶⁾ Affordable, high-end clinical diagnostic assays with robust operational performance in varied and suboptimal conditions are needed.⁽⁷⁾ Thus it is more challenging to develop POC diagnostics for resource-limited settings than resource-rich settings.⁽⁷⁾

More innovations in technology will follow in the coming years to address the challenges of POC diagnostics in resource-limited settings.⁽⁷⁾ Joint efforts from multidisciplinary research as well as collaboration with industry are required to speed up the development of POC diagnostics.⁽⁷⁾ With further refinement and clinical diagnostic trials, it is hoped that the diagnostic assays described in this thesis will eventually pave the way to improved POC TB tests for use in resource-limited settings.

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