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Utility of novel diagnostic tests for tuberculosis using human urine

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DECLARATION

I, Tapuwa Enwell Muchinga , 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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RESEARCH OUTPUT FROM THE PROJECT

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THESIS DEDICATION

This thesis is dedicated to my lovely wife, Prisca, who has always stood by me and offered invaluable motivation, support and encouragement throughout the entire research study.

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ABSTRACT

Background. Two thirds of new TB cases in sub-Saharan Africa are HIV co-infected. HIV-TB co-infection increases the incidence of extra-pulmonary, sputum smear-negative and sputum-scarce TB. In these vulnerable patient-groups with high mortality rates, sputum-based diagnostic tools are unhelpful. Urine-based diagnostics offer an attractive, easily available alternative for rapid diagnosis. We evaluated the point-of-care urine LAM strip test (Determine TB LAM Ag test, Alere) and urine-based Xpert MTB/RIF for TB diagnosis in two patient cohorts with high HIV prevalence.

Methods: A spot urine sample was collected from two cohorts of persons with suspected TB. The first cohort consisted of ambulatory primary care clinic patients suspected of having TB (group 1) whilst the second comprised hospitalised patients with suspected HIV co-infection (group 2). The urine LAM ELISA, LAM strip test and Xpert MTB/RIF were performed according to the manufacturer's instructions. In addition, the effects of using an alternative 'rule-in' cut-point for the urine LAM strip test and a pelleted (2-10ml) urine sample for Xpert MTB/RIF testing on diagnostic accuracy and inter-reader reliability was assessed. The diagnostic reference standard was *M. tuberculosis* culture positivity.

Results: Amongst group 1 out-patients, 28% (80/287) were HIV-infected and 14% (40/287) were *M. tuberculosis* culture positive. In group 1 HIV-infected patients using the grade-1 cut-point, the sensitivity (95%CI) and specificity (95%CI) of the LAM strip test was 24% (11-45) and 84% (73-92), respectively. Using the grade-2 compared to the grade-1 cut-point significantly improved test

specificity (84% vs 100%, p=0.02) and inter-rater agreement (84% vs. 100%, p=0.001) but sensitivity decreased to 5% (1-23).

Amongst group 2 hospitalised-patients, 83% (280/335) were HIV-infected and 42% (116/280) were *M. tuberculosis* culture positive. In HIV-infected patients the sensitivity (95%CI) of both urine LAM strip test (grade-2 cut-point) and urine-based Xpert-MTB/RIF was 48% (39-57). Combined sensitivity of urine LAM and Xpert MTB/RIF was better than each test alone [combined: 64% (55-73) vs. LAM strip test: 48% (39-57), p=0.014 vs. MTB/RIF: 48% (39-57), p=0.014]. In sputum-scarce, culture positive patients, urine-based Xpert MTB/RIF had a sensitivity of 40% (8/20). For Xpert MTB/RIF, the use of 2-10ml of pelleted urine vs. 1ml unprocessed urine in paired samples improved diagnostic yield [18% (14/38) vs. 0% (0/38), p<0.001].

Conclusions: Use of a grade-2 and not the manufacturer's suggested grade-1 cut-point for the urine LAM strip test yields optimal inter-reader agreement, test specificity and rule-in utility. In ambulatory primary care patients, urine LAM strip testing offers limited diagnostic utility. By contrast, in hospitalized HIV co-infection with advanced immunosuppression, especially those unable to produce sputum, both the urine LAM strip and Xpert MTB/RIF testing, alone or in combination, offers important potential clinical utility for rapid rule-in TB diagnosis. Further large prospective studies are needed to confirm these findings.

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ABBREVIATIONS

AFB	acid-fast bacilli
Ag	Antigen
AraLAM	arabinose-capped lipoarabinomannan
AIDS	Acquired Immunodeficiency Syndrome
BAL	broncoalveolar lavage
BCG	Bacille Calmette Guérin
CFU	Colony Forming Unit
CI	Confidence Interval
CSF	cerebro-spinal fluid
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
CNS	Central nervous system
CXR	Chest x-ray
ELISA	Enzyme-Linked Immunosorbent Assay
EPTB	Extrapulmonary tuberculosis
FNAB	Fine needle aspirate biopsy
GIT	Gastro-intestinal tract
HIV	Human Immunodeficiency Virus
LAM	Lipoarabinomannan
LOD	Limit of detection
LTBI	Latent tuberculosis infection
ManLAM	Mannose-capped lipoarabinomannan

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MDR TB	Multi-Drug Resistant tuberculosis
MSU	Midstream urine
M. tb	Mycobacterium tuberculosis
NAATs	Nucleic Acid Amplification Tests
NPV	Negative predictive value
NTM	Non-tuberculous mycobacteria
OD	Optical density
PCR	Polymerase Chain Reaction
PI	phospho-*myo*-inositol
PILAM	Phospho-*myo*-inositol capped LAM
PPD	Purified Protein Derivative
PPV	positive predictive value
POC	Point-of-care
PTB	Pulmonary tuberculosis
RIF	Rifampicin
ROC	Receiver Operating Characteristic
rpoB	RNA polymerase β
rRNA	ribosomal ribonucleic acid
SPC	Sample processing control
SOP	Standard operating procedures
SN	Sputum negative
SS	Sputum scarce
TB	Tuberculosis

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TNF	Tumour necrosis factor
TST	Tuberculin Skin Test
UCT	University of Cape Town
USA	United States of America
WHO	World Health Organisation
XDR TB	Extremely Drug Resistant tuberculosis
ZN	Ziehl-Neelsen

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CHAPTER 1: LITERATURE REVIEW

1.0. Introduction

The history of humankind has been inextricably linked with tuberculosis (TB), a communicable disease caused by *Mycobacterium tuberculosis (M. tuberculosis)*. Today, TB remains a public health catastrophe affecting the world's poorest and most vulnerable people. Anually, there are almost nine million new cases and two million deaths globally [1]. In sub-Saharan African the colliding HIV and TB epidemics continue to overburden hospitals and healthcare resources. The lack of new TB drugs or an effective vaccine together with the rising threat of multi-drug and extensively-drug resistant TB, threaten to destabilise the situation further [12, 13]. In resource-limited, high TB HIV prevalent settings suboptimal and/or delayed diagnosis is one of the major the bottleneck problems hindering TB control efforts. HIV, poverty, inadequate laboratory infrastructure and the lack of affordable and effective point-of-care diagnostic tools intersect to create this bottleneck of suboptimal and/or delayed diagnosis.

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1.1. An urgent need for TB point-of-care (POC) tests

In the majority of res In the majority of resource-limited settings, the frontline TB diagnostic tool remains sputum smear microscopy in both peripheral clinic and hospital settings. Front-loaded smear microscopy, considered a POC test, in settings of high HIV prevalence has reduced diagnostic accuracy. There is an urgent need to develop affordable, simple point-of-care (POC) methods that are effective in resource-limited, high TB HIV prevalent settings. Recommended specifications for a TB POC test are shown and summarised in table 1.0 [11, 14]. POC tests should be cost effective, non-invasive, requiring minimal laboratory

skills, rapid and applicable in the resource-limited settings such as Sub-Saharan Africa.

Table 1.0 Some of the minimum specifications for TB POC diagnostic tests that need urgent development [10, 11].

Test Specification	**Minimum Required Value**
Medical decision	Treatment initiative
Time to results	3 hours max. (patient must receive results the same day) *[Desirable would be <15min]*
Throughput	20 tests/day, minimum, by 1 lab staff
Specimen type	Adults: urine, oral, breath, venous blood, sputum *[Desired: NON sputum-based sample type and use of finger prick instead of venous blood]* Children: urine, oral, capillary blood (finger/heel prick)
Sample preparation	- 3 steps max. - Safe: biosafety level 1 - Ability to use approximate volumes (ie, no need for precise pipetting) - Preparation that is not highly time sensitive
Number of samples	One sample per test
Readout	- Easy-to-read, unambiguous, simple "yes", "no", or "invalid" answer - Readable for at least 1 hour
Waste disposal	- Simple burning or sharps disposal; no glass component - Environmentally acceptable disposal
Controls	- Positive control included in test kit - Quality control simpler and easier than with SSM
Reagents	- All reagents in self-contained kit - Kit contains sample collection device and water (if needed)
Storage/stability	- Shelf life of 24 months, including reagents - Stable at 30°C, and at higher temperatures for shorter time periods (to be defined) - Stable in high humidity environments
Instrumentation	- If instrument needed, no maintenance required - Instrument works in tropical conditions - Acceptable replacement cost - Fits in backpack - Shock resistant
Power requirement	Can work on battery
Training	- 1 day max. training time - Can be performed by any health worker
Cost	<US$10 per test after scale-up (Consensus could not be reached on a definite minimum value.)

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This study evaluates urine-based diagnostic tests that have a potential to function as TB POCs in immune-suppressed HIV/TB coinfected patients who are either hospitalised or ambulant patients reporting to peripheral clinics in resource limited settings such as the Sub-Saharan Africa.

1.2. POC test characteristics for different settings

An ideal POC test should be both a good rule-in and rule-out test. However, no TB test is yet to meet these requirements for different forms of TB disease and different clinical settings [10]. Consequently, certain test characteristics are more suitable to different clinical settings and levels of the healthcare system. For instance, in peripheral, primary care clinics where patients present with less advanced TB disease, patient volumes are very high, and available confirmatory tests are expensive and time-consuming e.g. TB culture, a POC test with good rule-out value (i.e. a screening test) is required [11]. This would allow for confident, rapid screening and discharge of non-TB patients together with the triaging of patients for doctors' review and screening with diagnostic tools with better specificity [11]. By contrast, in hospitalized patients with more advanced disease burden where the need to rapidly and appropriately commence treatment is paramount, a rapid rule-in POC test would be preferred [15]. A good rule-in test, with high specificity would prevent the unnecessary administration of potentially toxic therapies, while at the same time allowing for early commencement of life-saving treatment [15].

1.3. TB Epidemiology and the impact of HIV

The global distribution of new TB and HIV cases are shown in figure 1.1 and 1.2. South Africa has the fifth highest incidence of TB globally and approximately 60% of new TB patients are co-infected with HIV [16].

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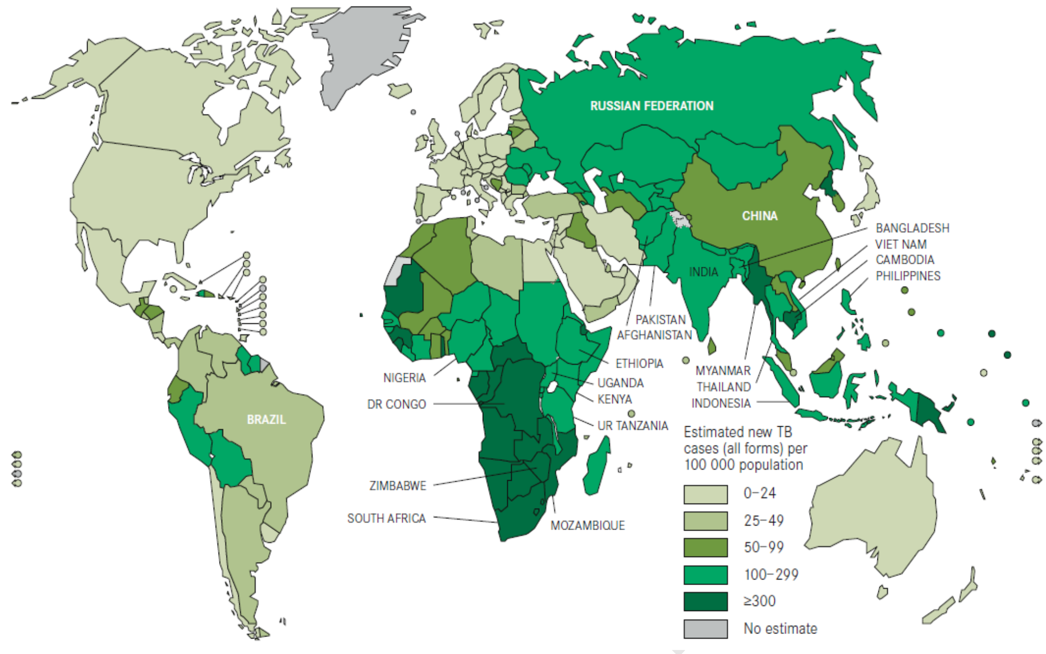

Figure 1.1. Estimated TB incidence rates, 2010 [1].

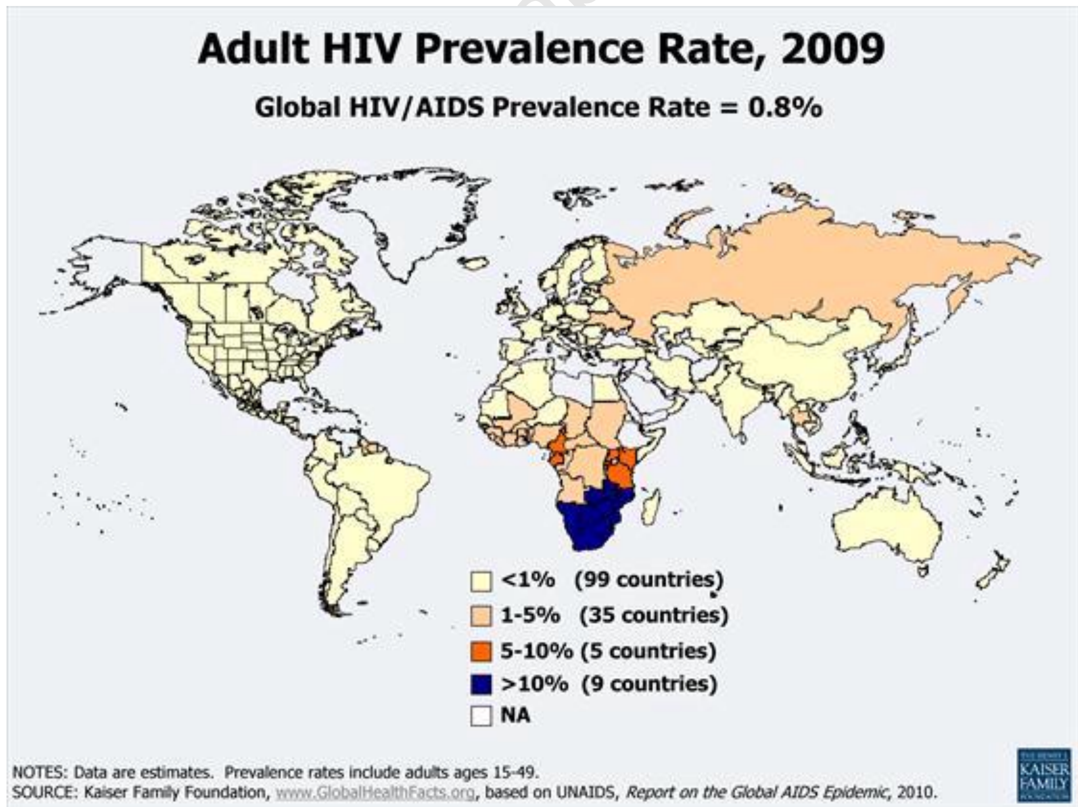

Figure 1.2. HIV prevalence in adults, 2009 [2].

The colliding epidemics of HIV and TB in sub-Saharan Africa profoundly affect the clinical management and public health control of both conditions [17]. HIV-infected persons, regardless of level of immunosuppression, are at increased risk for developing active TB [16-18]. TB-related mortality is higher amongst HIV co-infected people, and TB remains the leading cause of death amongst HIV-infected people [19]. With advancing immunosuppression, the clinical presentation of TB becomes atypical with an increase in smear-negative, extra-pulmonary and disseminated forms of TB disease [13]. Consequently, TB diagnosis using conventional diagnosis is particularly challenging amongst HIV-infected persons and sub-optimal [20].

1.1. Sputum-based TB diagnostics in resource-limited and high HIV-prevalent settings

1.1.1. Smear microscopy

Smear microscopy remains the cornerstone of TB diagnosis in the majority of high burden settings. It is a quick, inexpensive, and specific method for TB diagnosis with a sensitivity of up to 80% in immunocompetent patients [21]. Unfortunately, it has several drawbacks including: i) decreased sensitivity (as low as 20%) in HIV co-infected patients, ii) very low performance in pauci-bacillary EPTB (<5% sensitivity in pleural, CSF and pericardial fluids), and iii) does not allow for drug susceptibility testing [21, 22]. Despite extensive efforts to optimize smear microscopy, including the use of concentration, bleaching, fluorescent staining and LED microscopy, performance remains sub-optimal in high HIV prevalent settings [23].

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1.1.2. Chest x-ray (CXR)

Chest x-ray (CXR)is often the only adjunctive TB diagnostic tool available to clinicians at hospitals in high burden settings, and is often used to make empiric treatment decisions. However, the sensitivity and specificity of CXR for TB diagnosis in different settings is highly variable. Several factors such as HIV status, primary clinic or hospital setting, experience of CXR reader and even the sex of the patient contribute to the variability of the CXR performance [24]. Notably, in HIV co-infected patients who are severely immune-compromised the radiographic appearances of TB can be atypical or absent, with one study showing up to 32% of active TB cases with a normal CXR [23]. Furthermore, the poor specificity of CXR means that a number of patients are inappropriately treated with anti-TB treatment with intendant morbidity. The need for a definitive microbiological diagnosis remains.

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1.1.3. *M. tuberculosis* culture

M. tuberculosis culture (Figure 1.3), is considered the reference standard diagnostic tool for TB [25]. Some studies across Africa showed that culture can detect active pulmonary tuberculosis (PTB) and extra-pulmonary TB that was shown to be negative by smear microscopy [26]. However, the culture method is limited by the extremely slow growth rate of the *M. tuberculosis*, taking 4-6 weeks for results to be available, thereby limiting its utility for clinical decision-making and/or delaying the commencement of treatment [27]. Additionally, in HIV-infected patients often presenting with pauci-bacillary disease, the diagnostic yield of a single culture is reduced [25].

Fig.1.3. Culture on Lowenstein-Jensen medium revealed typical dry, heaped-up yellow to buff-coloured colonies of *Mycobacterium tuberculosis* [3].

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Finally, the performance of culture requires adequate laboratory infrastructure and trained microbiology personnel to limit contamination rates [28]. Laboratory infrastructure and human resources remain limited in resource poor high TB burden settings [6].

1.1.4. Novel molecular diagnostics

Polymerase chain reaction (PCR) methods that identify specific sequences of *M. tuberculosis* DNA or rRNA are exciting new tests for TB diagnosis and are known as nucleic acid amplification tests (NAATs). A number of commercial NAATs are widely available and utilised for TB diagnosis. PCR has been , as early as 1991, to be very useful in amplifying and detecting DNA fragments of *M. tuberculosis* from uncultures clinical samples such as cerebral spinal fluid (CSF) and urine [29]. A study by Partolli et al (1991), demonstrated that

PCR had much higher sensitivity than any other method, such as microscopic visualization or DNA hybridization that were used in the direct detection of *M. tuberculosis* [29]. However, PCR involves the use robust infrastructure that requires highly sterile environments, highly skilled personnel and also highly bio-hazard material. Nevertheless, another study by Orallo et al () also demonstrated the useful and potential role of PCR in the detection of M.tuberculosis using EPTB samples such as CSF, pleural fluid, synovial fluid, pericardial fluid, urine, bone marrow aspirate, tissue biopsies etc {Rhoda Lynn C. Orallo, 2008

#1018}. In this study they showed that although PCR had higher sensitivity than most of the methods in the diagnosis of TB, the method had low Specifity that was associated with false results from non-tuberculous infections and other contaminants {Rhoda Lynn C. Orallo, 2008

#1018}. A more rapid user-friendly realtime PCR that can be used with little skill and reduced bio-hazard is now available yet producing results with higher sensivity and specificity.

1.1.5. Xpert MTB/RIF

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Xpert MTB/RIF assay is a real-time PCR platform with excellent sensitivity, specificity and low indeterminate rate which can provide a result in less than two hours. It is the most exciting new TB diagnostic developed in decades. The Xpert MTB/RIF assay integrates DNA extraction, genomic amplification, and semi-quantitative detection of *M. tuberculosis* complex and rifampicin (RIF) resistance into a fully automated system [25, 30, 31]. Table 1.1 highlights the superior diagnostic accuracy of Xpert MTB/RIF compared to smear microscopy to the diagnosis of pulmonary TB [32]. Based on this data, in December 2011, the World Health Organisation (WHO) endorsed the Xpert MTB/RIF assay for use in the investigation of pulmonary TB and MDR-TB. The WHO made strong recommendations that Xpert MTB/RIF should be

used for frontline TB diagnosis in HIV-infected patients and MDR-TB suspects [32]. On world TB day last year, the South African Health minister announced that South Africa will replace smear microscopy with the Xpert MTB/RIF assay for frontline TB diagnosis [33].

Table 1.1. Summary of Xpert MTB/RIF sensitivities in comparison to smear microscopy. The findings were based on demonstration studies involving 6,673 individuals prospectively enrolled in six distinctly different settings

	SENSITIVITY	
Group of patients tested	**Xpert MTB/RIF (%)**	**Sputum smear microscopy (%)**
Single smear/culture positive patients	99	59.5
Overall culture positive patients	91	59.5
		47 in HIV coinfection
Smear negative patients	>80	n/a
HIV positive TB patients	86	47
HIV negative TB patients	92	65
***MDR confirmed patients (Rif resistance)**	95.1	n/a

*Specifity was 98.4% rifampicin detection

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However, despite the promising results of the sputum-based Xpert MTB/RIF, it does not solve a number of important diagnostic challenges and many obstacles remain to its widespread implementation.

1.1.5.1. Xpert MTB/RIF and EPTB

Xpert MTB/RIF is dependent on an adequate sputum sample and is hence, ineffectual in sputum scarce patients [6]. Limited available data suggests that Xpert MTB/RIF has sub-optimal sensitivity in non-sputum biological fluid for the diagnosis of EPTB, although no studies have adequately evaluated its performance using urine [8, 34]. Table 1.2 shows some of the studies on Xpert MTB/RIF use in EPTB and its sensitivies and specificities. Most of the studies

shown in table 1.2 that assessed the use Xpert MTB/RIF in EPTB made use of EPTB specimens that were obtained from non-pulmonary sites from which samples are invasive, bio-hazardous and difficult to extract. A few studies evaluate performance on urine specimens and numbers of patients are small [8, 35]. Hence, there is an urgent need for a larger study to evaluate the effectiveness of urine Xpert MTB/RIF in the diagnosis of EPTB. Furthermore, despite efforts to reduce test cost, Xpert MTB/RIF remains unaffordable in many high burden resource-limited settings [6, 36]. There remains a need for more affordable, preferably point-of-care and non-sputum based TB diagnostic tools.

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Table 1.2 shows a summary of the studies in which the diagnostic accuracy of Xpert MTB/RIF for EPTB was assessed [37] .

Study	Country	EPTB samples	Xpert sensitivity	Xpert specificity	Ref.
Tortoli *et al.* (2012)	Italy	Tissue biopsies/fine-needle aspirates (94); pleural fluid (18); gastric aspirates (61); pus (55); CSF (14); urine (16); peritoneal/ synovial/pericardial fluid (10)	81.3 (76.2–85.8)	99.8 (99.4–100)	[35]
Armand et al. (2011)	France	LNs (16); pleural (7); bone (5)	53.1 (34.7–70.9)	NA	[38]
Causse *et al.* (2011)	Spain	Tissue biopsies (18); CSF (6); gastric aspirates (8); pleural fluid (4); purulent exudates (5)	95.1 (83.5–99.4)	100 (98.8–100)	[39]
Friedrich *et al.* (2011)	South Africa	Pleural fluid (25)	25.0 (8.7–49.1)	100 (47.8–100)	[40]
Hillemann *et al.* (2011)	Germany	Tissue (30); gastric aspirate (8); urine (5)	77.3 (60.5–87.1)	98.2 (96.0–98.9)	[8]
Ligthelm *et al.* (2011)	South Africa	Fine-needle aspiration LN biopsy	96.6 (86.6–100)	88.9 (69.6–100)	[41]
Moure *et al.* (2011)	Spain	All smear-negative. Pleural fluid (26); LNs (34); abscess aspirates (17); tissues (12)	58.3 (48.5–67.8)	100 (91.4–100)	[30]
Vadwai et al. (2011)	India	Tissue biopsies (105); pus (98); body fluids (24)	80.6 (75.5–85.0)	99.6 (97.8–100)	[42]

1.2. Extrapulmonary Tuberculosis (EPTB) and HIV-coinfection

EPTB is TB disease affecting organs other than the lungs, and the most common forms include body cavity (pleural, pericardial and ascitic), lymph node, and meningeal [43] . EPTB is responsible for 10-20% of global TB cases and has increased substantial in areas of high HIV prevalence as the incidence of EPTB and disseminated forms of TB increase with worsening immunosuppression [13, 44].

The diagnosis of EPTB is particularly difficult and is the most important obstacle to improved management. Both conventional and novel sputum-based diagnostic tools such as smear microscopy, Xpert MTB/RIF and *M. tuberculosis* culture have reduced diagnostic accuracy [44]. Consequently, diagnosis often requires invasive, expensive tissue sampling for histologically diagnosis and to improve the likelihood of microbiological confirmation [45]. Table 1.3 gives a summary of the major forms of EPTBs indicating the nature of specimen needed for diagnosis and current frontline diagnostic method being used by most settings especially resource-constrained settings. Empiric treatment based on clinical and radiological screening is commonplace in the management of EPTB, and novel non-sputum based diagnostics and diagnostic strategies are urgently required [45].

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Table 1.3. Summary of the major forms of EPTB and their possible diagnostic methods in use in most settings.

Type of EPTB	Percentage cases of EPTB	Site of Infection	Nature of specimen	Possible diagnostic methods *(PCR required in all cases- if available)*
Tuberculous lymphadenitis [46]	35% [43]	-Lymph nodes -Discrete and firm nodes	Fine needle aspiration biopsy (FNAB)	-Acid fast bacillus stain (AFB) (Zielh-Nelseen stain or Papanicolaou stain induced florescence microscopy) [41] Culture of FNAB
Skeletal TB [47] 1-3% of TB population	10% [43]	-joints, bones -tuberculous arthritis e.g. Of hip and knee -vertebral column [48]	-FNAB -bone biopsy	-ZN stain microscopy and Culture of FNAB [48]
TB of the central nervous system (CNS) [46]	5% [43]	-intracranial -meninges tissue -spinal chord	-Cerebral spinal fluid (CSF)	-CSF AFB smears -CSF *M. tuberculosis* culture -Pelleting of CSF increases culture and smear results.
Abdominal TB [46]	3% [43]	-peritoneal, gastro-intestinal tract (GIT), liver, spleen etc.	-histopathological biopsies	-AFB smear and *M. tuberculosis* culture of biopsy tissue.
Miliary TB [49]	8% [43]	-widespread dissemination throughout the body due to delays.	-Broncho alveolar lavage (BAL), gastric washings, blood, liver biopsy, bone marrow, peritoneum etc. [49]	-Culture and all other diagnostic methods
Pericardial TB [43, 46]	1-2% [50]	-visceral or parietal pericardium.	-Pericardial fluid, pericardial biopsy	- AFB smears, ZN staining on pericardial biopsy, TST, Biochemical analysis, *M. tuberculosis*culture on biopsy, PCR.
Pleural TB [43, 46, 51]	20% [43]	-Pleural fluid, pleural space.	-Pleural fluid, pleural biopsy, fragments of peritoneum.	-Cytological examination (ZN and Giemsa stain), PCR, TST, *M. tuberculosis* culture on biopsy (after centrifugation), biochemical analysis.
TB ascites [43, 46]	2% [52]	-Ascitic fluid, peritoneal cavity	-Ascitic fluid, peritoneal biopsy	-Cytological examination (ZN and Giemsa stain), PCR, TST, *M. tuberculosis* culture on biopsy, biochemical analysis
Genito-urinary TB [44]	9% [43]	Kidney, urinary tract, bladder, etc.	-urine	-AFB smear, *M. tuberculosis* culture.

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1.3. Urine for TB diagnosis

Urine as a biological sample for diagnostic testing is appealing. Urine is easy to collect, readily available and has a low infection risk to staff during collection [53]. Urine antigen detection is the most common diagnostic technique employed for infectious diseases, and TB diagnosis is no exception [54]. A number of *M. tuberculosis* antigens have been evaluated in urine for the TB diagnosis [54]. Of these 12 evaluated TB antigens, lipoarabinomannan (LAM) is the most extensively evaluated and promising [54].

1.3.1. Urine lipoarabinomannan (LAM) for TB diagnosis

LAM is a heat-stable 17.5kD glycolipid that forms one of the main components of the outer cell wall of mycobacterial species, and is a heterogeneous immune-reactive glycoconjugate [4]. It accounts for up to 15% of the total bacterial weight and is an important virulence factor of *M. tuberculosis* [55-57]. LAM consists of three distinct structural domains, including a phosphatidylinositol (PI) anchor, a branched mannan, and a branched arabinan [58]. Figure 1.4 shows the *M. tuberculosis* cell wall structure highlighting the position of LAM.

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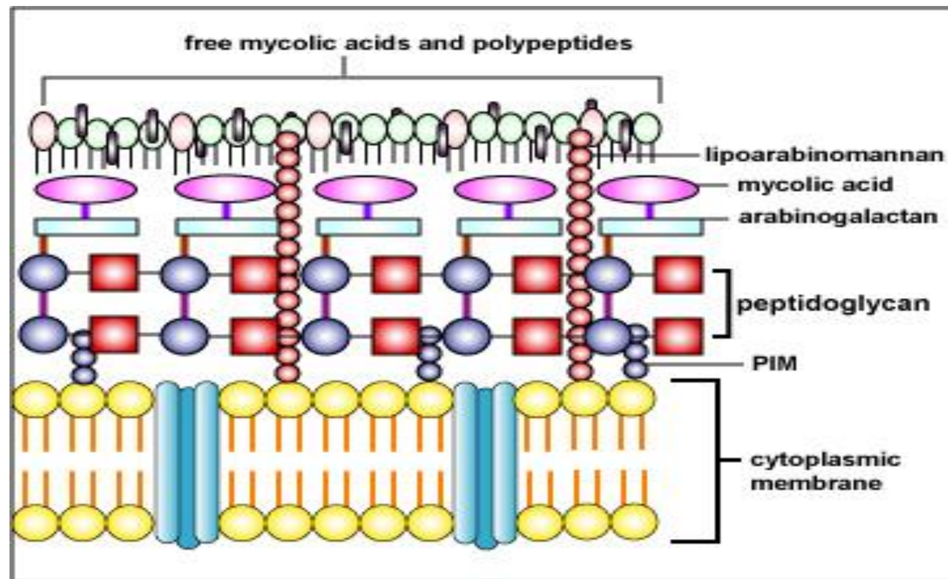

Fig 1.4. A simplified illustration of the *M. tuberculosis* cell wall showing Lipoarabinomannan [4].

There are three types of LAM namely mannose-capped LAM (ManLAM), phospho-*myo*-inositol capped LAM (PILAM) and non-capped arabinose LAM (AraLAM) [59]. The different types of LAM capping determines the ability of LAM to modulate immune responses; ManLAM (simply referred to as LAM) is the commonest form in *M. tuberculosis* and is a very potent anti-inflammatory molecule and virulence factor [59]. The mannose caps, as recently discovered, may be involved not only in attenuating host immune response, but also in mediating the binding of mycobacteria to and subsequent entry into macrophages [60]. ManLAM is postulated to modulate a number of host responses including: cytotoxic oxygen free-radical scavenging; inhibition protein kinase C activity and; prevent the interferon gamma transcription in macrophages and T-cells. The inhibition of macrophage activation, abrogation of T-cell activation and blockage of the cytocidal activities contributes immensely to the persistence of *M. tuberculosis* within the mononuclear phagocytes and their dissemination to other parts of the body. This shows that ManLAM, which also possesses much less potency in evoking TNF-alpha and other responses (compared

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to other types of LAM) is an immunogenic virulence factor of much clinical and diagnostic significance [61, 62].

1.3.2. Detection of LAM in the urine

LAM can be detected in the urine of patients with active TB. This heat-stable glycolipid travels in the bloodstream and passes through the renal filtration barrier without major changes and is thus detectable in an antigenically intact form in urine [5, 9, 63] (see Figure 1.5).

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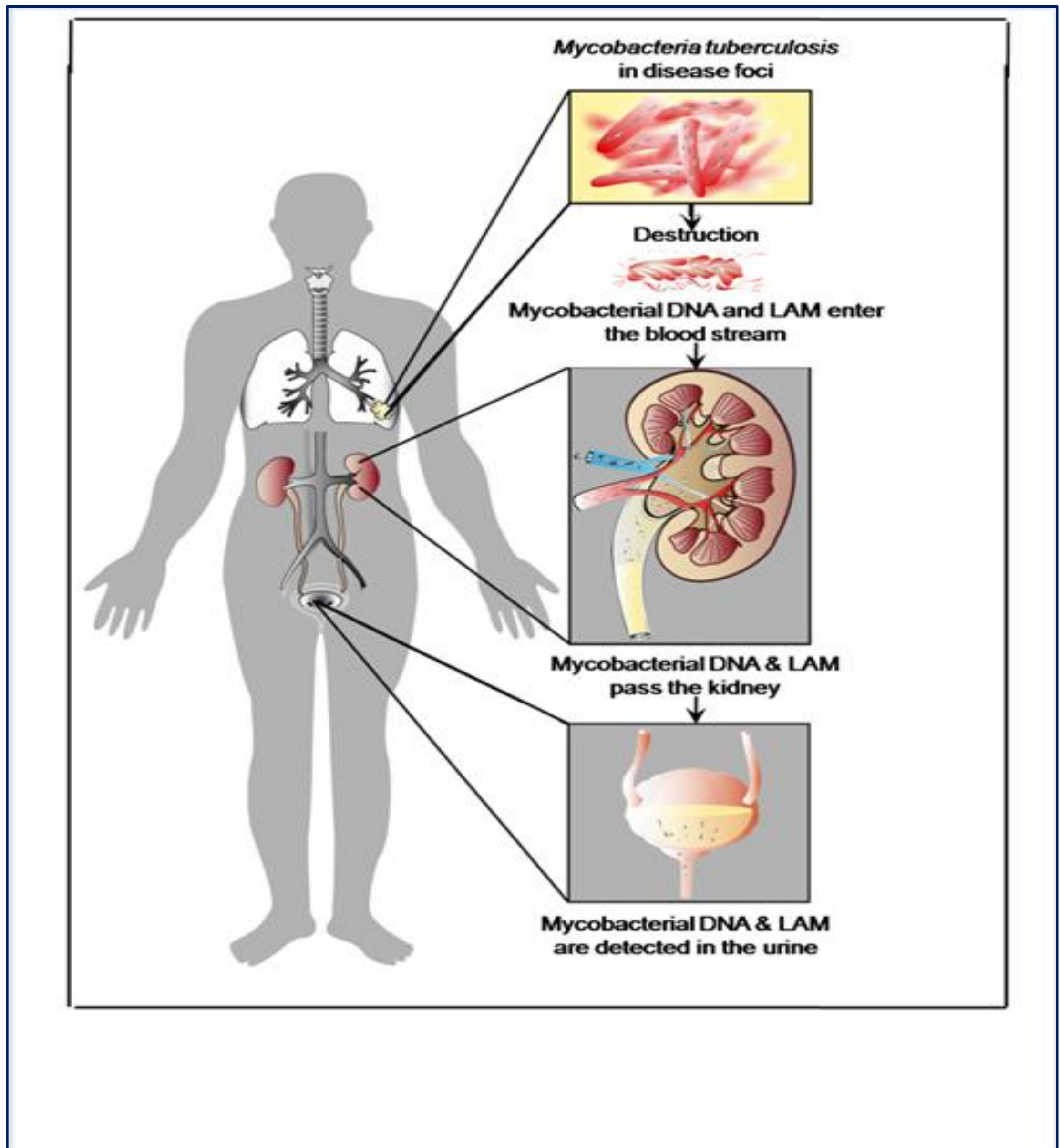

Figure 1.5. Annotated diagram illustrating the passage of mycobacterial DNA and lipoarabinomannan antigen from infection site to Urine LAM [4].

Hamasur and colleagues developed an ELISA detection method using a polyclonal antibody. Preliminary studies showed that LAM was detectable in mice urine injected intraperitoneally with a crude cell wall preparation of *M. tuberculosis* and the methods were highly sensitive, detecting LAM at concentrations of 1 ng/ml and 5 pg/ml [63]. In 2005, the pre-commercial prototype of the LAM ELISA (*M. tuberculosis* LAM ELISA Test®, Chemogen, and Portland, USA) was first tested in human urine in Tanzania. This pre-

commercial version was superceded by a second generation, commercially-available prototype known as the Clearview® TB ELISA, (Inverness Medical Innovations, USA). The test currently retails as the TB LAM ELISA (Alere, USA).

1.3.3. LAM ELISA test performance

The TB LAM ELISA-kit has been extensively evaluated for the diagnosis of TB. The majority of studies evaluate performance using urine as the biological sample of choice [4]. However, researchers have, with little success, investigated the diagnostic utility of the LAM ELISA on other biological fluids like serum [64], sputum [65], cerebro-spinal fluid (CSF) [66] and pleural fluid [65, 67]. Table 1.4 outlines the evaluations of the urinary LAM ELISA to date. Apart from the early studies of by Boehme and Tessema [5, 68], the overall sensitivity of urine LAM ELISA has been poor ,ranging from 13-51%, for the routine diagnosis of TB [9, 67, 69, 70] . However, amongst HIV-infected patients with advancing immunosuppression (lower CD4 cell counts) and hospitalized in-patients with higher burdens of disease, urine LAM ELISA sensitivity improves [4]. Shah *et al*. (2009), in a South Africa hospitalised cohort study showed a sensitivity of 85% in TB HIV co-infected patients with a CD4<50 cells/ml [71]. In addition, and of particularly clinical importance, in this same study the sensitivity of urine LAM amongst sputum smear-negative patients was 56% (Table 1.4) [71]. Thus, urine LAM testing seems to offer the greatest potential utility in the diagnosis of TB in HIV co-infected patients with advancing immunosuppression [45]. This is particularly useful given that sputum-based conventional and novel diagnostic tools perform poorly, EPTB and disseminated TB is more common and TB-related mortality propotions are up to 50% [46, 72].

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Table 1.4. Performance of standardized urine lipoarabinomannan assays in different clinical subgroups of patients with suspected tuberculosis stratified by HIV status and, where available, CD4 T cell count [4].

Study	Clinical subgroup and country	Evaluable patients tested/ Total number recruited	HIV (%pos.)	Overall sensitivity(CI), HIV pos. patients with CD4< 200µl/ml		Overall specificity(CI), HIV pos. patients with CD4< 200µl/ml	Overall PPV(CI), HIV pos. patients with CD4< 200µl/ml	Overall NPV(CI), HIV pos. patients with CD4< 200µl/ml	Sensitivity (CI) smear(S) neg. culture(C) pos. TB cases (n) (n= S-ve, C+ve)
Boehme et al. [3]	Outpatient clinic and healthy USA/local controls	235/333	69.0%	OVERALL	80	99 (N/R)	100 (N/R)	80(N/R)	(n=50) 76 (N/R)
				HIV+	81	N/R	N/R	N/R	N/R
				CD4<200	N/R	N/R	N/R	N/R	N/R
Daley et al. [65]	Outpatient clinic (India)	200/200	8.5%	OVERALL	18 (9-33)	88 (81-92)	30 (15-50)	79 (72-84)	(n=12) 25 (4-64)
				HIV+	20 (1-77)	83 (51-97)	33 (2-88)	71(42-90)	N/R
				CD4<200	N/R	N/R	N/R	N/R	N/R
Reither et al. [8]	Outpatient clinic (Tanzania)	151/291	59.1%	OVERALL	51 (38-63)	88 (79-94)	78 (63-89)	68 (58-77)	(n=21) 38 (N/R)
				HIV+	62 (47-75)	84 (68-94)	84 (68-94)	62(47-75)	42 (N/R)
				CD4<200	N/R	N/R	N/R	N/R	
Mutetwa et al. [66]	Outpatient clinic (Zimbabwe)	261/397	77%	OVERALL	44 (36-54)	89 (81-94)	84 (74-91)	54 (46-61)	(n=40) 28 (13-43)
				HIV+	52 (43-62)	86 (73-93)	84 (73-92)	57 (48-66)	N/R
				CD4<200	N/R	N/R	N/R	N/R	N/R
Dheda et al. [59]	Outpatient clinic (South Africa)	427/500	31%	OVERALL	13 (8-19)	99 (97-100)	94 (74-99)	59 (53-64)	(n=70) 3 (N/R)
				HIV+	21 (11-35)	100 (91-100)	100 (70-100)	55 (43-65)	18 (N/R)
				CD4<200	37 (19-59)	100 (84-100)	78 (45-94)	76 (63-86)	25 (N/R)
Lawn et al. [67]	ARV clinic, asymptomatic HIV patients (South Africa)	235/235	100%	OVERALL	38 (27-51)	100 (N/R)	100 (82-100)	83 (77-88)	(n=50) 36† (N/R)
				HIV +	38 (27-51)	100 (N/R)	100 (82-100)	83 (77-88)	
				CD4 50-100	41(22-64)	N/R	N/R	N/R	36† (N/R)
				CD4< 50	67 (44-84)	N/R	N/R	N/R	35† (N/R
Shah et al. [68]	In-patients (South Africa)	315/499	85%	OVERALL	59 (52-66)	96 (91-99)	73 (65-80)	34 (29-39)	(n=111) 56 (N/R)
				HIV+	67 (59-74)	94 (87-98)	75 (67-82)	30 (25-36)	
				CD4 50-100	71 (51-87)	N/R	N/R	N/R	N/R
				CD4 <50	85 (73-93)	N/R	N/R	N/R	N/R
Gounder et al [69]	Outpatient clinic (South Africa)	422/443	100%	OVERALL	32 (16-52)	98 (96-99)	53 (28-77)	95 (93-97)	(n=19) 16 (4-40)
				HIV+	32 (16-52)	98 (96-99)	53 (28-77)	95 (93-97)	(n=19) 16 (4-40)
				CD4 <200	35 (16-57)	96 (92-99)	57 (29-82)	92 (87-95)	N/R
				CD4 <50	56 (21-86)	90 (76-97)	56 (21-86)	90 (76-97)	N/R
Talbot et al [70]	In-patients (Dares Salaam, Tanzania)	212/278	100%	OVERALL	65 (53-76)	86 (79-91)	69 (56-80)	84 (76-89)	(n=24) NR
				HIV+	65 (53-76)	86 (79-91)	69 (56-80)	84 (76-89)	(n=24) NR
				CD4 50-200	67 (43-85)	94 (79-99)	88 (60-98)	82 (66-92)	N/R
				CD4 <50	77 (58-89)	72 (56-84)	67 (49-81)	82 (65-92)	N/R

1.4. The LAM lateral flow strip test

Excitingly, Alere Medical innovations has recently developed a simple lateral flow format of the Clearview TB LAM ELISA using the same polyclonal antibody coupled to gold nanoparticles [6, 19]. The Determine-TB LAM Ag strip test (Alere, USA) (figure 1.8), the first truly point-of-care TB test, requires just 60µl of unprocessed urine, can be performed at the patient bedside without the need for laboratory skills or electricity and at a likely cost of less than US$3.50 [6]. Urine is placed on to the loading platform, and after just 25mins the test is read using the manufacturer's provided reference card to interpret band intensity. The presence of a band in the control window indicates a valid test, and then the colour intensity of the band in the patient window is matched to the reference card and graded intensity 0-5 (figure 1.8). Thereafter, the manufacturer and reference card categorises patients with a grade 0 bands as test 'negative' and a grade-1 or higher band as test 'positive'. This simple test offers great potential for resource-limited high TB HIV burden settings.

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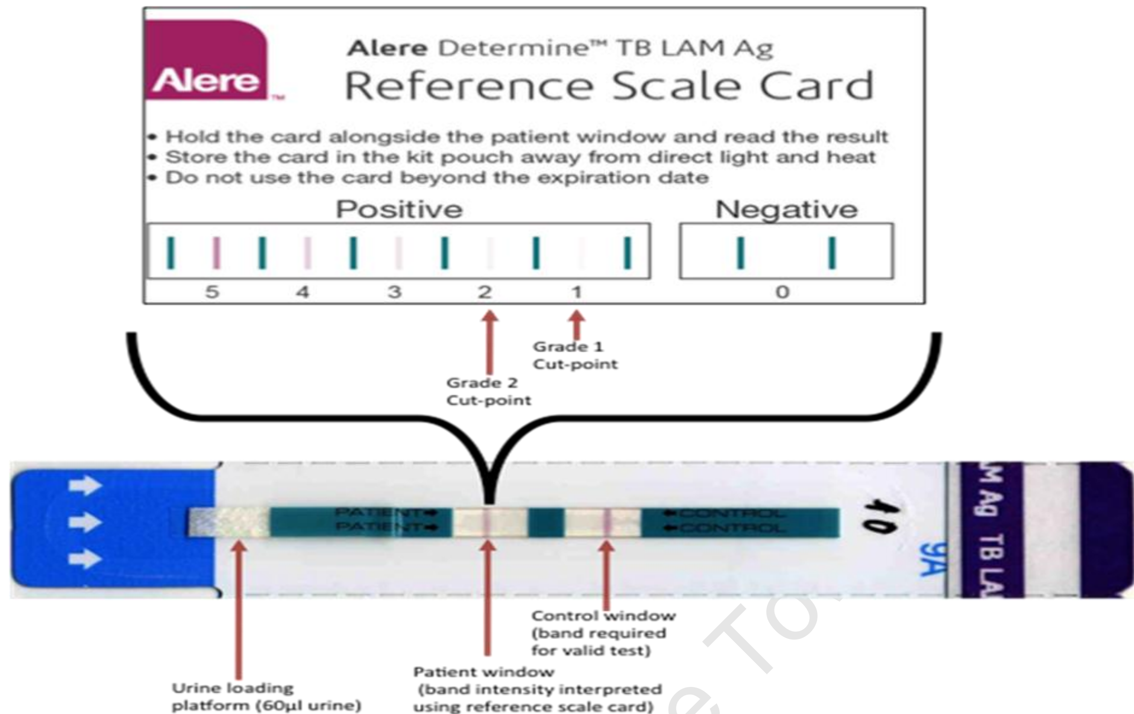

Figure 1.8. The LAM strip and reference scale card (graded 0-5 depending on band intensity) that were used in the reading of urine LAM results. Grade-1 cut-point considers '0' as negative and 1-5 as positive while grade-2 cut-point takes '0 and 1' as negative and 2-5 as positive [6].

1.4.1. Urine LAM strip test initial diagnostic accuracy evaluation

To date, the urine LAM strip test has only been evaluated in two studies. The first in an out-patient antiretroviral clinic setting where the test was used for TB screening patients prior to the initiation of antiretroviral therapy irrespective of symptoms [19], while the second was a cohort of hospitalised patients with suspected TB HIV co-infection [6].

1.4.2. Urine LAM strip test for TB screening pre-HAART

The evaluation of the Determine TB-LAM test strips by Lawn et al (2012) in outpatients that were being screened for antiretroviral therapy at a primary care setting in South Africa showed very promising results [19, 69]. Overall and

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using the manufacturer-recommended suggested grade-1 cut-point; they found a sensitivity and specificity of 28.2% and 98.6% respectively. Sensitivity consistently improved with advancing immune-suppression, increasing to 39% and 51.7% in patients with CD4<200 and CD4<100 cells/ml respectively [19]. As expected, urine LAM strip test diagnostic accuracy was found to be similar to that of the urine LAM ELISA assay performance [69, 71]. Furthermore, the inter-reader reliability between two readers was found to be excellent (K=0.97), but it was noted that the median LAM ELISA optical density of urine LAM strip positive samples was 0.681 (IQR 0.164–2.431; range 0.102–3.291) meaning that few grade-1 positive samples were present in the patient cohort [19]. The effectiveness and utility of the LAM strip test is dependent on its high specificity which other studies working with the ELISA version showed [67, 73]. The performance and utility of the LAM strip test was also evaluated by other studies and was found to be promising.

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1.4.3. Urine LAM strip test in hospitalised TB HIV co-infection

Peter *et al* (2012) recently evaluated the diagnostic accuracy of the urine LAM strip test for TB detection in hospitalised patients with suspected TB HIV co-infection and severe illness. Overall using the manufacturer's suggested grade-1 cut-point and a composite reference standard, urine LAM strip sensitivity and specificity was 66% and 96% respectively. However, inter-reader reliability was found to be only moderate using the grade-1 cut-point and in addition, amongst a non-TB control group, test specificity and positive predictive value (important for rule-in test utility) was reduced using the grade-1 versus the grade-2 cut-point [6]. Hence, this study recommended the use of a grade-2 and not the grade-1 cut-point to optimize the rule-in utility of the test [6]. Sub-group analysis indicated that the urine LAM strip test performs best in patients with lower CD4 cell counts and significantly outperformed smear microscopy in the

diagnosis of EPTB [6]. Furthermore, in the clinically important smear-negative or sputum scarce (SN/SS) HIV-coinfected patient sub-group the urine LAM strip test sensitivity was 38% increasing to 49% in patients with CD4≤ 100 cell/ml [6].

Both the out-patient and in-patient studies of the urine LAM strip test showed promising results, but important differences were found. Most notably, the optimal urine LAM strip test cut-point requires further clarification. Possible explanations for the differences in study findings include study populations differences, batch variability, reader experience and/or blinding procedures, and duration and stability of LAM strip tests during storage [6].

1.4.4. LAM strip test as and adjunctive diagnostic test

Urine LAM strip testing is unable to offer microbiological confirmation and/or drug susceptibility testing. Furthermore, studies of urinary LAM show only modest to good overall sensitivity even amongst HIV-infected patients with advanced immunosuppression [6, 19, 45]. Thus, urine LAM strip testing will likely function as a diagnostic adjunct to existing, largely sputum-based conventional and novel rapid TB diagnostics such as smear-microscopy or Xpert MTB/RIF [45]. In line with this rationale, both Peter *et al* (2012) and Lawn *et al* (2012) evaluated the combined performance of urine LAM and either sputum smear-microscopy or Xpert MTB/RIF [6, 19]. Interestingly, in both out-patient and in-patient cohorts the combined sensitivity of sputum smear-microscopy and urine LAM strip testing was significantly higher than the use of each test alone [6, 19]. In addition, Lawn *et al* (2012) found that combined sputum-based Xpert MTB/RIF and urine LAM strip testing was not significantly better than sputum Xpert MTB/RIF alone [19]. However, the sub-group of sputum scarce patients, in whom sputum-based diagnosis is unhelpful, was reduced by the use

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of sputum induction to aid sample acquisition. Sputum induction is not readily available in the majority of primary care clinic settings [6, 45]. The use of urine-based Xpert MTB/RIF in sputum scarce patients was not evaluated in either study.

1.5. Is the urinary LAM specific for *M. tuberculosis*?

Notwithstanding that the urinary LAM is the most promising point-of-care (POC) diagnostic test; there still remains the major setback of cross reactivity with other microorganisms that have LAM-like glycolipids, thereby increasing the possibility of false positive LAM results. Although LAM is a lipopolysaccharide specific to the genus *Mycobacteria,* it is interesting that LAM-like glycolipids are also found in several species of fungi and bacteria and these are likely to cross-react with LAM antibodies [74].

1.5.1. Possible cross-reactions with polyclonal anti-LAM antibody

Several studies have shown and reported that NTMs and other microorganisms can cross-react with polyclonal anti-LAM antibody leading to the LAM assays failing to be specific for *M. tuberculosis* only [67]. Thus far, Boehme *et al.* (2005) has shown *in vitro* that the LAM ELISA can cross react with non-tuberculous mycobacterial (NTM) species although higher NTM colony counts are required to produce LAM ELISA optical density above the cut-point for test positivity [5]**.** Gram-positive and Gram-negative samples were prepared with bacterial concentrations higher than those in the normal infections yet none showed any reactivity in the LAM-ELISA system [5]. Boehme *et.al* (2005) pre-clinical evaluation of the LAM-ELISA using a variety NTMs and other bacteria is shown in figure 1.6 below.

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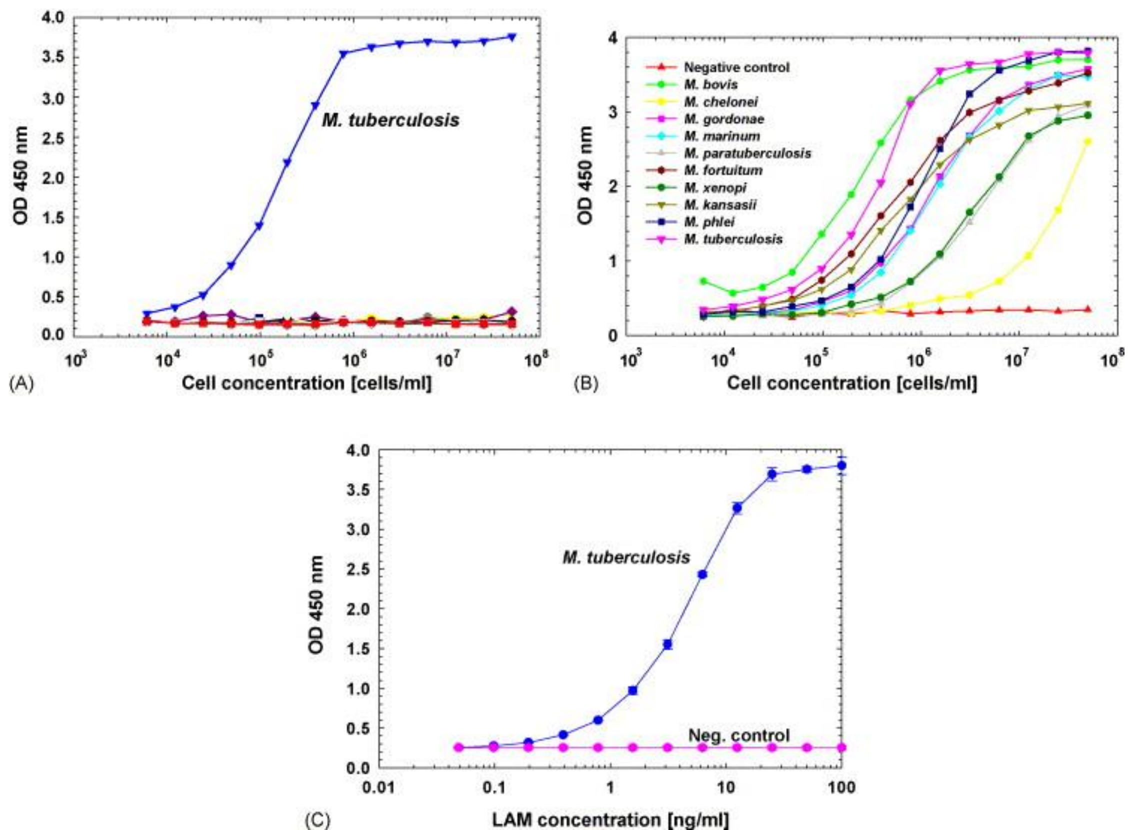

Figure1.8. (A) No binding of LAM antibodies to Gram-positive and Gram-negative bacteria: *Klebsiella pneumoniae, Streptococcus agalactiae, Streptococcus pneumoniae 14/12F, Pseudomonas aeruginosa, Staphylococcus aureus 25923/43300, Proteus vulgaris, Escherichia coli 8739, Neisseria meningitidis A/B/13102, Haemophilus influenzae A/B/D*. (B) Reactivity of the listed NTMs in the LAM-ELISA. (C) Sensitivity of LAM-ELISA for increasing concentrations of LAM in urine [5].

G. bronchialis demonstrated presence of substantial LAM-like lipoglycan arabinose content and showed cross-reactivity with polyclonal anti-LAM antibody [75]. Furthermore, Dheda et.al (2010) carried studies on the possibility of mouth flora cross-reacting with polyclonal LAM antibodies in the LAM ELISA assay [67]. They used sputum samples from non-TB patients and tested cultures and culture supernatants of the specific organisms composing both pathogenic and normal mouth flora, especially those possessing LAM-like lipoglycans in their cell walls e.g. Nocardia and Streptomyces species (strain-typed where possible), *C.albicans, T. paurometabolum*, and *Candida neoformans* [67]. The results which they obtained are shown in Figure 1.7. The study demonstrated that Clearview® TB ELISA had a high sensitivity in sputum

samples but the specificity was very low possibly because of cross-reactivity with LAM-like microbial lipopolysaccharides in the cell walls of several mouth-residing organisms such as Candida (also found in genitals), and many species of Actinobacteria [67].

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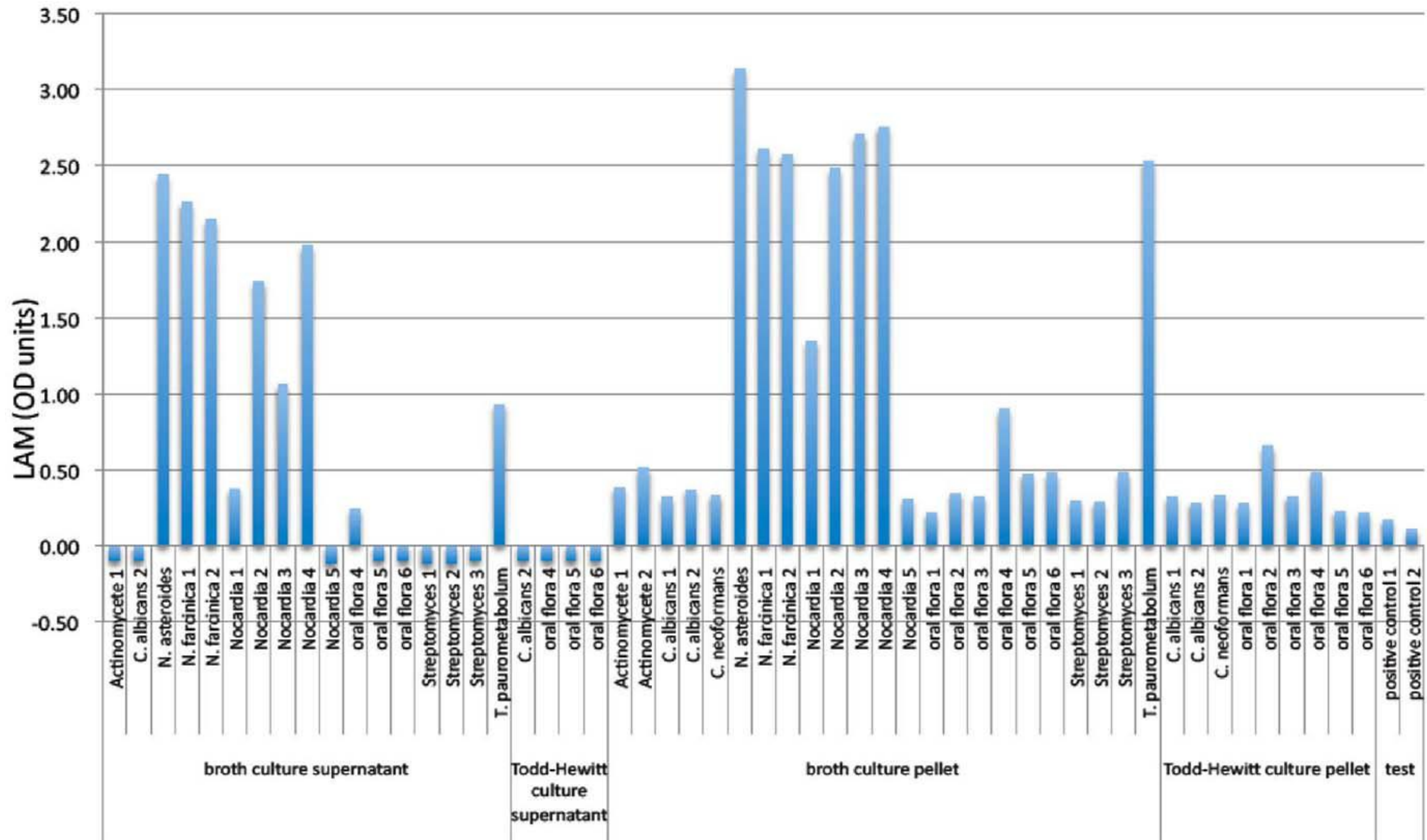

Figure1.7. Dheda et al (2010) demonstrated LAM positivity in cultures of oral flora and in organism-specific cultures such as various Actinobacteria, including different strains of Nocardia and Streptomyces, and C. albicans, T. paurometabolum, and C. neoformans [54].

These NTMs and other opportunistic pathogens such as Candida species could cross-react with the polyclonal LAM antibodies within the assay [54]; thereby likely to culminate into false positive LAM ELISA results. This creates the need for the development of a standardized method for urine sample collection to maintain sample sterility; for this will significantly decrease the rate of false positive urine LAM ELISA/lateral flow tests.

1.5.2. Use of a standardized urine sample collection methodology

Since a false-positive urine LAM test is likely to be caused by environmental contamination or unrelated infections (colonization or disease by non tuberculous mycobacterial or fungi), there is a need to establish a standardized urine sample collection methodology [67]. This is important, and is done in order to maintain sample sterility, thus it will likely significantly decrease the rate of false positive urine LAM ELISA/lateral flow tests. The importance of preventing contamination by normal vaginal, perineal and anterior urethral flora cannot be overemphasized and remains the responsibility of the laboratory to provide the patient with sterile, wide-mouthed glass or plastic, jars, beakers or other suitable receptacles which should have tight-fitting lids [76]. Suprapubic aspiration urine collection method is the gold standard; unfortunately the method is not practical in many cultural settings. However, whenever possible, urine specimen should be collected in the morning, before the patient has voided urine and ensure that mid-stream urine or a clean-catch urine is collected [76].

There is several urine collection methods that are in use to date that strive to eliminate any possibility of contamination thereby improving the specificity of the LAM assays. Table 1.5 below shows the WHO guideline on standardised sterile urine collection procedures that can be followed by

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many health care settings to in order to minimize risk of contamination of urine samples.

Table 1.5. The WHO Regional Office for South-East Asia (2011) listed the following guidelines on SOPs for Microbiology [70].

WHO guideline on standardised sterile urine collection procedures*
(The sample is best obtained, if the procedure is properly explained to the patient)
Urine collection in men
Instruct the patient to wash hands.
Ask the patient to pull back the foreskin and pass a small amount of urine holding back the fold of skin; instruct the patient to pass the remaining urine in a sterile container – this is mid-stream urine (MSU).
Place the lid, secure tightly and rapidly transport to the laboratory.
If the patient is bed-ridden, the nursing staff can help the patient in the above process.
Urine collection in women
Instruct the woman to wash hands with soap and water before collection of specimen.
Patient should undress in a suitable room, spread the labia and cleanse the vulva and labia thoroughly using warm soapy water.
Rinse with warm water and dry.
Ask the patient to pass urine, discarding the first part of the stream and collecting MSU in a sterile container.
Transport the sample to the laboratory at the earliest after properly securing the lid.
Bed- ridden patients can be assisted by the nursing staff.
Urine collection in in Infant and young children
Ask the child to drink water or any other liquid.
Clean the external genitalia and let the child be seated in the lap of the mother/nurse/attendant.
Encourage the child to urinate and collect the same in sterile container.
Cover the container tightly and rapidly transport to the laboratory for processing.

**Collection of urine from catheters or bag should be avoided as this does not reflect the accurate picture. A reasonable alternative to MSU is the clean catch urine. After periuretheral cleaning the whole urine is collected into a sterile container and then an aliquot is sent for examination* [76].

1.7. Current study: Aims and objectives

The overall objective of this project is to evaluate the use of two novel tools for the TB diagnosis using human urine. In an ambulatory patient cohort (group 1), the diagnostic accuracy and inter-observer agreement of two independent readers for the urine LAM strip test is evaluated in order to clarify the optimal cut-point. In a hospitalised patient cohort, the combined diagnostic accuracy of urine-based Xpert MTB/RIF and LAM is evaluated.

1.7.1. Ambulatory patient cohort (group 1)

Hypothesis: The use of a urine LAM strip grade-2 cut-point instead of the manufacturer's recommended grade-1 cut-point will significantly improve inter-rater agreement and specificity thereby optimizing rule-in diagnostic utility.

Aim 1: To evaluate inter-observer agreement between 2 independent test readers grading the urine LAM strip tests.

Aim 2: To assess the diagnostic accuracy of the urine LAM strip test using the manufacturer's suggested grade-1 and an alternative grade-2 cut-point.

1.7.2. Hospitalised HIV-infected cohort (group 2)

Hypothesis: Amongst hospitalized HIV-infected patients with advanced immunosuppression, the combination of urinary LAM and Xpert MTB/RIF will offer important, incremental and potentially rapid diagnostic utility.

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Aim 1: To evaluate the combined diagnostic performance of the urine LAM strip test or LAM ELISA and urine Xpert MTB/RIF in HIV co-infected patients with suspected PTB and/or EPTB and advanced immunosuppression.

Aim 2: To evaluate the combined diagnostic performance of the urine LAM strip test, LAM ELISA and urine Xpert MTB/RIF in sputum smear-negative and sputum scarce culture-positive, HIV co-infected patients with suspected PTB and EPTB and advanced immunosuppression.

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CHAPTER 2: METHODS AND MATERIALS

2. **Methods and materials for the two study arms**

2.1. Introduction

This study evaluated the diagnostic accuracy of three novel TB diagnostic tools in human urine: Urine LAM lateral flow strip test (Determine TB®), Xpert MTB/RIF assay (Cepheid GeneXpert® System and Clearview® TB ELISA, (Inverness Medical Innovations, USA). These diagnostics were studied in two patient cohorts: ambulatory patients presenting with TB symptoms to three primary care clinics (group 1), and hospitalised patients with suspected TB HIV co-infection and advanced immunosuppression (group 2). The diagnostic accuracy of each tool, alone or in combination, was evaluated using sensitivity, specificity, positive and negative predictive values (PPV, NPV). Inter-reader agreement was assessed by a comparison of proportional agreement and kappa statistics.

2.2. Data collection and management

The integrity of this whole study dependent immensely on the way in which data was collected. A lot of effort and quality control were placed into every stage of data collection right from the clinics and hospital to the laboratory where most of the diagnostic work was done.

The study involved urine samples as specimens for research. Patient recruitment and clinical data collection were done by consultant-led groups of attending clinicians, with no association to the study team. This helped in the reduction of errors emanating from biases. Research nurses who helped in the collection of data were well trained. All the data collected at the recruitment sites were carefully recorded in a spreadsheet and immediately sent to the data capturers of the Lung Infection and Immunity Unit who took professional care of all the research data from the clinicians. Urine samples were collected and processed as described

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later, but all the laboratory procedures on the urine samples and data collection were done by well-trained senior laboratory research scientists who were blinded to the clinical details and disease conditions of the patients. Well-designed forms were used to immediately record results and all the data from the laboratory diagnosis was recorded and stored in a Microsoft Excel spreadsheet and Microsoft Word was used to generate tables for further data storage. Not less than two senior scientists were involved in the analysis of the results especially the LAM strip test procedures. All this was done to ensure that the quality control of data was maintained and followed.

2.2.1. Group 1 prospective cohort patients

The group 1 patient cohort consisted of a consecutive series of 300 ambulant self-reporting TB suspects (≥18 years of age) presenting to Gugulethu clinic, a primary health care facility in Cape Town, South Africa (Figure 2.1). Gugulethu Township is a predominantly black African disadvantaged community that has a very high HIV prevalence. Patients were prospectively enrolled if they had symptoms suggestive of pulmonary TB including: persistent fevers, loss of weight, night sweats, lack of appetite, generalized fatigue, chest pain and a cough for greater than 2 weeks. Enrolled patients were also, after appropriate counselling and encouragement, tested for HIV and CD4 count and their results were determined and recorded. Demographic, physical and clinical data was taken and recorded for each patient on validated and detailed case record forms. A written and signed informed consent was obtained from each patient and the study was approved by the University of Cape Town Human Research Ethics Committee.

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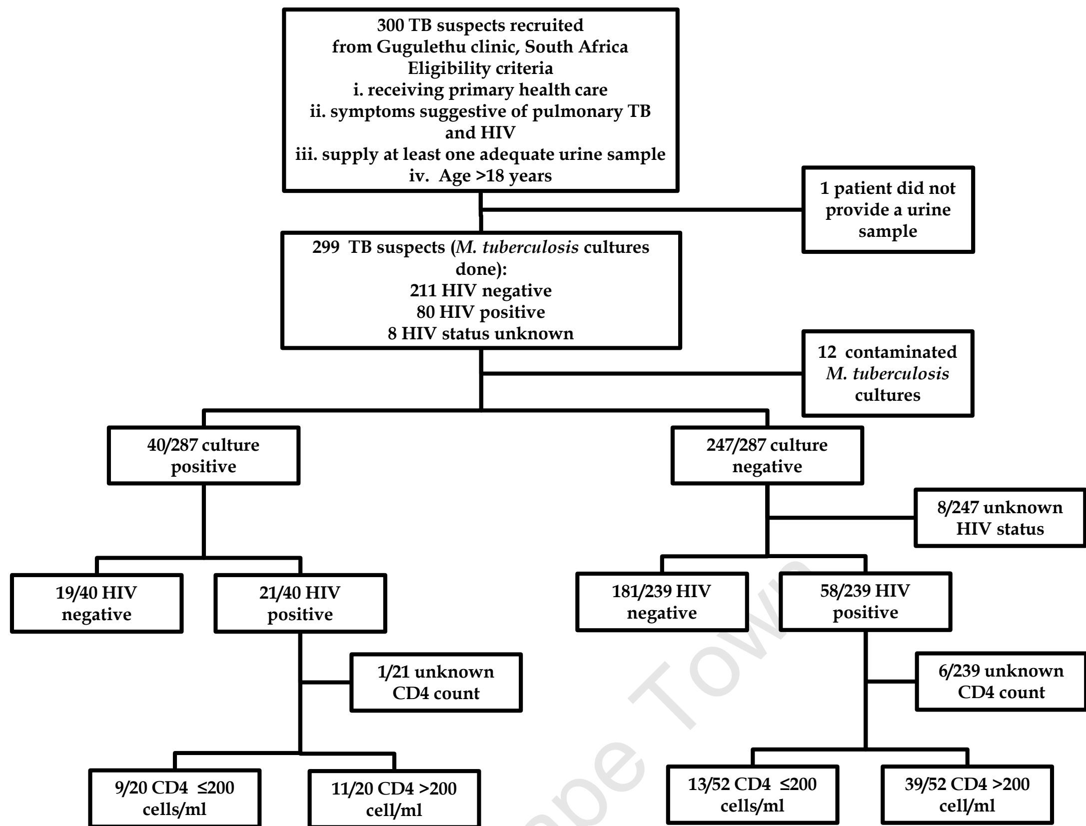

Figure 2.1. Study flow for group 1 patient cohort showing TB diagnostic, HIV status and CD4 count stratification.

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2.2.2. Urine collection and storage

A spot urine sample (10-30ml) was collected in a sterile container from each patient as soon as possible after recruitment. A urine dipstick test (UriCHECK 9, RapiMed Diagnostics, South Africa) was immediately performed to assess for protein, blood and leucocytes. All the urine samples that were collected from each subject were transported to the Lung Infection and Immunity Unit within 3-4 hours for further testing. Urine for LAM strip testing was immediately transferred from the sterile urine pots into sterile 2ml aliquot and was stored at -20°C for later batched testing.

2.2.3. Laboratory methods

The urine samples used in the study were stored at -20°C and at the time of LAM strip testing the samples were thawed and mixed or vortexed for approximately 5 seconds. After mixing the urine, 60µl were carefully pipetted onto the loading bay or sample pad of the LAM strip after the removal of the protective foil. After 25 minutes, the LAM strips read/graded under ambient laboratory lighting conditions, avoiding direct sunlight. All strips were graded between 25-35 minutes after urine was loaded onto the platform. Two readers, blinded to the clinical details of the patients, were involved in the reading and interpretation of results. A third reader was present to independently read the LAM strips and the results were used as tie-breakers in the case of discrepancies in grading between the first two readers, thereby reaching a consensus. A manufacturer-provided visual reference scale card accompanied each pack of 100 LAM strips to assist readers with the grading of coloured bands. Figure 2.2 shows the LAM strip and reference scale card that were used in the reading of urine LAM results. The presence of a band in the control window indicates that the test is valid; thereafter the presence/absence of a band in the patient window is examined.

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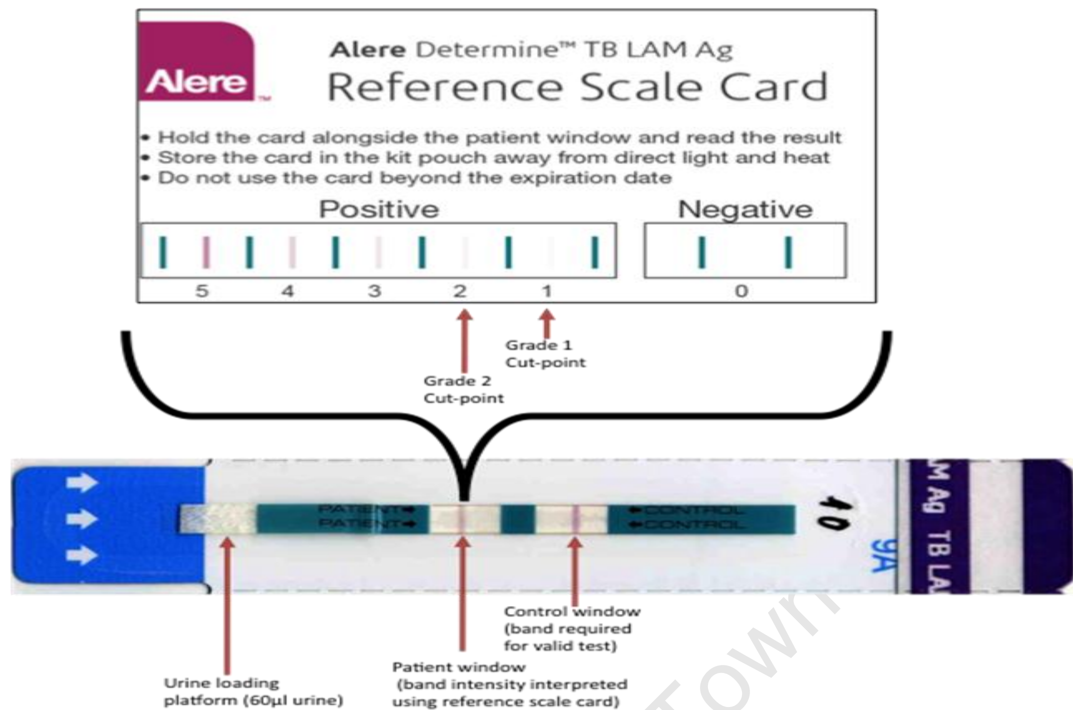

Figure 2.2. The LAM strip and reference scale card (graded 0-5 depending on band intensity) that were used in the reading of urine LAM results [6].

2.2.4. Grading of LAM strip results

The colour intensity of a bar in the patient window was graded 0-5 using a scale reference card as shown in figure 2.2. The manufacturer recommends a grade-1 cut-point, which means that the appearance of any complete band (grade 1-5) is interpreted as "test positive" while the complete absence of a band is interpreted as "test negative". A broken or incomplete bar in the patient window, irrespective of band intensity, is interpreted as a "test indefinite" result. The use of the alternative grade-2 cut-point suggested by Peter *et al* (2012) means that the appearance of only a grade 2-5 band is interpreted as "test positive", while both the complete absence of a band and a grade-1 band is interpreted as "test negative" [6].

Peter et al selected the grade-2 cut-point to optimise rule-in test utility using receiver operating characteristic (ROC) curve analysis shown below in Figure 2.3 [6].

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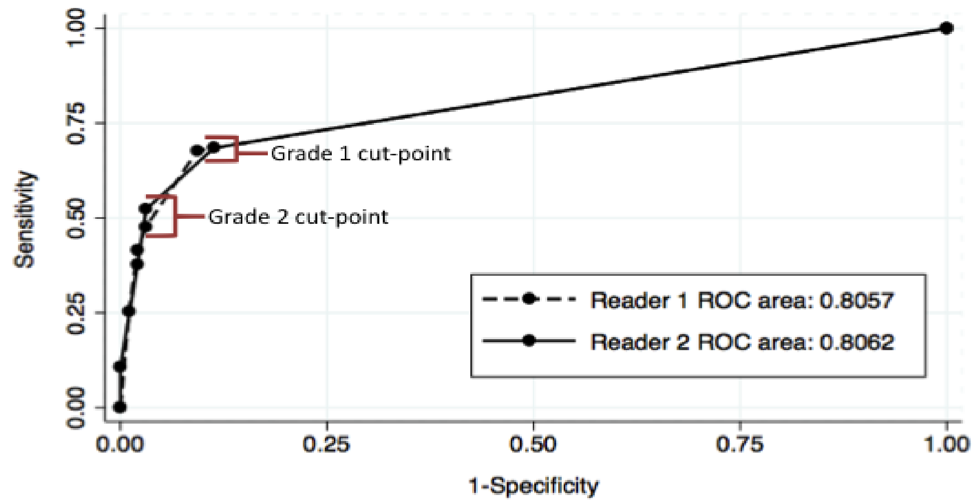

Grade 1 cut-point	Reader 1	Reader 2
Sensitivity	65.7% (88/134)	68.3% (95/139)
Specificity	89.2% (91/102)	89.7% (104/116)
Indeterminate* rate (after repeating inconclusive tests once)	1.5% 4/273	0.7% 2/273
Positive Likelihood ratio	6.1	6.6

Grade 2 cut-point	Reader 1	Reader 2
Sensitivity	46.3% (62/134)	50.4% (70/139)
Specificity	97.1% (99/102)	97.4% (113/116)
Indeterminate* rate (after repeating inconclusive tests once)	1.5% 4/273	0.7% 2/273
Positive Likelihood ratio	15.7	19.5

Figure 2.3. The receiver operator characteristic (ROC) curves that Peter *et al* (2012) used in the LAM strip test grading conducted by 2 independent readers. Sensitivity, specificity, indeterminate rate and likelihood ratios for visual band intensity grade-1 cut-point (manufacturer-recommended) and grade-2 cut-point (optimal 'rule-in') are indicated [6].

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2.2.5. Statistical measures of agreement

In order to interrogate the differences in inter-reader agreement for the urine LAM strip testing using different cut-points, both a simple proportional agreement. Table2.1 below shows a categorisation scale that is frequently used to interpret the kappa statistic [77].

Table 2.1. The kappa values and their interpretations.

Interpretation of Kappa	
Kappa value	**Agreement**
<0	Less than chance agreement
0.001-0.20	Slight agreement
0.21-0.40	Fair agreement
0.41-0.60	Moderate agreement
0.61-0.80	Substantial agreement
0.81-0.99	Almost perfect agreement
1.00	Perfect agreement

2.2.6. Group 2 prospective cohort patients

Group 2 study population consisted of a consecutive series of 335 prospectively recruited adult patients that were recruited from four hospitals (3 district- and 1 tertiary-level hospital) between 22 July 2009 and 14 December 2010 in Cape Town, South Africa. There 3 patients who refused consent and were therefore excluded from the study. This group of patients consisted of hospitalized persons with suspected TB HIV co-infection (≥18 years of age) presenting to these four district hospitals in Cape Town, South Africa (Figure 2.4). Patients were enrolled if they had symptoms suggestive of pulmonary TB including: persistent fevers, loss of weight, night sweats, lack of appetite, generalized fatigue, chest pain and a cough for greater than 2 weeks, and either were known HIV-infected or clinically suspected to be. Enrolled patients were, after appropriate counselling and encouragement, tested for HIV and CD4 count and their results were determined and recorded. Demographic, physical and clinical data was taken and recorded for each patient on validated and detailed case record forms. A written and signed informed consent was obtained from each

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patient and the study was approved by the University of Cape Town Human Research Ethics Committee.

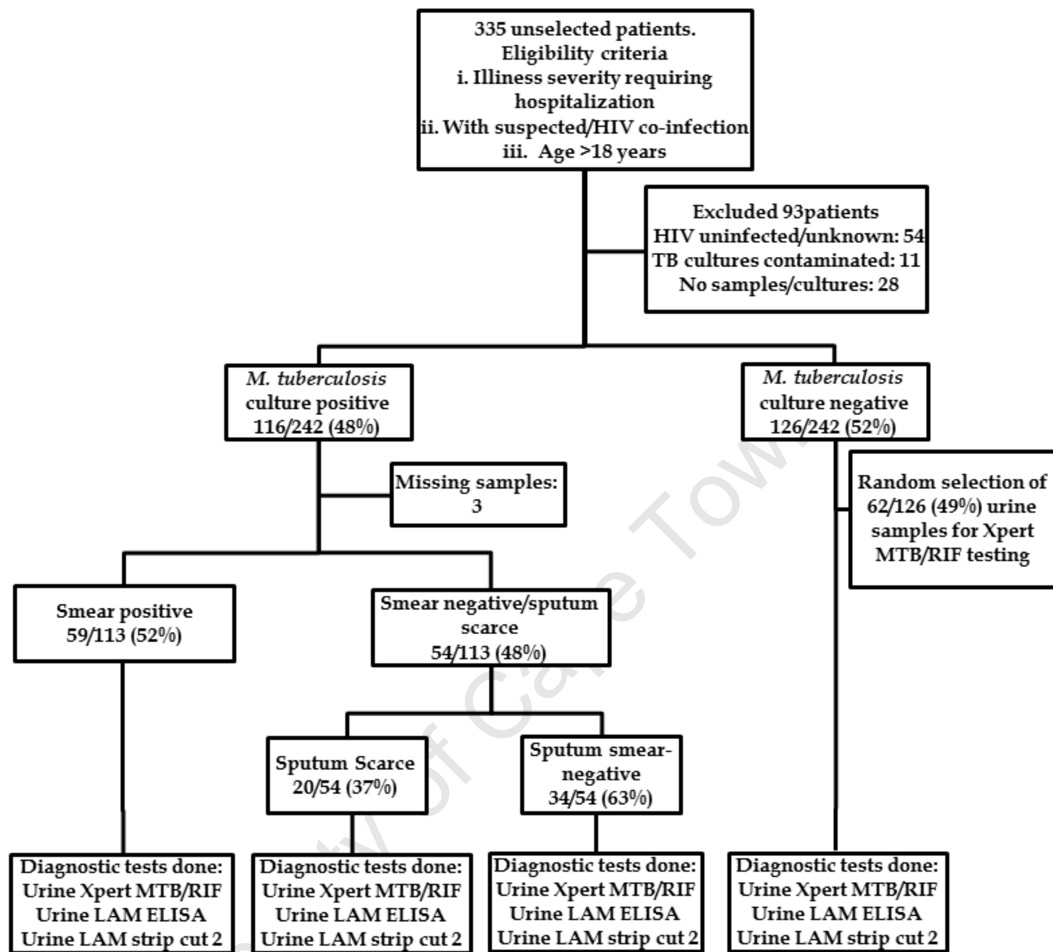

Figure 2.4. Outline of the study population groups and their breakdown into different TB diagnostic.

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2.2.7. Laboratory methods

2.2.7.1. Diagnostic sample collection handling

The timing and extent of the diagnostic work-up, the beginning of empiric anti-TB treatment and the final discharge of patients from the hospital were based upon decisions made by consultant-led groups that were composed of attending clinicians with no association to the study team. However, the TB diagnostic work-up was not standardised, but the routine local hospital practice was done; this included collecting two sputum samples in patients who were able to expectorate. Nevertheless,

if EPTB was suspected, 1-2 non-sputum samples were collected from the patients particularly from their clinically involved sites. The site from which the EPTB specimens were collected included fine needle aspirate of lymph node, pleural fluid aspirate/biopsy, ascitic tap, CSF, pericardial aspiration gastric washings and urine. The National Health Laboratory Services, a local reference laboratory, processed all clinical specimens collected for TB diagnosis. Fluorescence smear microscopy was performed on NALC/NaOH processed sputum, which was also cultured using the MGIT 960 liquid culture system (BD Diagnostics, USA). The reference standard for definite-TB was liquid culture positivity for *M. tuberculosis* [45].

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2.2.7.2. Urine sample collection and preparation

The urine samples that were used in the urine Xpert MTB/RIF assay were collected from patients recruited from the 4 hospitals in Cape Town, transported to the Lung Infection and Immunity Unit where they were stored and frozen in the -20°C refridgerator. A total of 175 urine samples, were collected and prepared to be run on the Xpert MTB/RIF assay; 113/175 (~65%) samples from *M. tuberculosi*s culture positive patients and 62/126 (~50%) were randomly selected urines from *M. tuberculosis* culture negative patients as shown in figure 2.4. The selection of urine from *M. tuberculosis* culture negative non-TB patients was random and only about half the total number of the available *M. tuberculosis* culture negative urine samples were coolected and prepared for Xpert; this was due to resource and time-constraints. This formed our control group which included HIV-infected TB-culture negative patients which allowed for the evaluation of urine Xpert MTB/RIF specificity. The details of the procedure for the collection and sample management are described in section 2.1.2.

However, the actual procedure and preparation of samples for the Xpert MTB/RIF assay are described in the next section 2.1.7.1. Some of the urine samples were run straight in the Xpert MTB/RIF assay without centrifuging because of the paucity of the urine sample available. Urine

samples that had adequate volumes for centrifuging were processed as described in the next sect section.

2.2.7.3. Urine sample pelleting for Xpert MTB/RIF assay

There were 175 urine samples, in total, that were run on the Xpert MTB/RIF assay, 113 samples from *M. tuberculosi*s culture positive patients and 62/126 randomly selected urines from *M. tuberculosis* culture negative patients. Only urine samples that were between 2ml-10ml were considered for pelleting; a total of 43/113 (41%) negative urine samples were selected for pelleting, while 13/113 (12%) negative urine samples were either insufficient or depleted. The urine samples were first mixed thoroughly and a specific volume (see Table 2.3), depending on the amount of urine available, was transferred to the blue capped 15 ml centrifuge tubes. Centrifugation of urine samples was performed at 3500 x g for 15 minutes in the ROTOFIX 32A (Hettich, zentrifugen) or BECKMAN COULTER (Allegra™ X-12R Centrifuge). Table 3.2 shows a summary of all the negative samples that were pelleted. After centrifuging the supernatant was removed using a PIPET.AID® automated pippete (Drummond Scientific Co. PA, USA) leaving 1 ml of urine in the tube for the resuspension of the pellet. The pellet was resuspended in the remaining solution and the 1 ml suspension was used for the Xpert MTB/RIF assay using the manufacturer’s instructions as described in figure 2.5. The rest of the supernatant and remaining urine samples were stored at -20°C.

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Table 2.3. The number and volume of urine samples that were pelleted by centrifuging at 3500 x g/ 15 minutes.

Volume of urine pelleted (ml)	*M. tuberculosis* culture-positive urine samples pelleted (n=43)	*M. tuberculosis* culture-negative urine samples pelleted (n=30)
2	2	0
3	1	0
5	8	0
6	1	1
7	1	0
7.5	0	1
8	1	1
8.5	1	1
10	28	26

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2.2.7.4. Urine Xpert MTB/RIF assay

Principle: The Xpert MTB/RIF assay (Cepheid GeneXpert® System) , shown in figure 1.4, is a hemi-nested real-time PCR in-vitro diagnostic test that is capable of detecting *M. tuberculosis* complex DNA in a sample while simultaneously detecting rifampicin resistance including a semi-quantitative assessment of bacillary load. The 15 minute-long step of mixing the bactericidal buffer with the sample is the only hands-on step of the whole process; this pre-amplification phase renders the *M. tuberculosis* non-viable and harmless as shown in figure 2.5 [78]. All these qualities of the Xpert MTB/RIF assay makes it a choice diagnostic tool for near patient usage especially in settings with poor bio-containment facilities [78, 79]. This process makes use of five overlapping molecular probes complementary to the whole *M. tuberculosis* rpoB gene containing the 81 base pair rifampicin resistance-determining region (RRDR) and they cover

all the mutations found in >99.5% of all rifampicin resistant strains ensuring that there no cross-reactivity with non-tuberculous mycobacteria occurs [32] . If there are at least two of these rpoB probes that are positive within two cycles of each other, then the result will be *M. tuberculosis* positive; also if at least a single rpoB probe does not result in a measurable signal and/or a presence of a 3.5 cycle or a significant deviation in the Cycle threshold (Ct) value between the earliest and latest rpoB signals then the system regards that as a RIF resistance result [80, 81]. An estimation of 95% of RIF resistant cases arise as a result of mutations found in the RRDR [36, 39]. This hemi-nested PCR amplification assay, integrated into a single disposable cartridge, depends on six colour fluorescent molecular beacons to detect the presence of any amplified target [36, 80]. The Xpert MTB/RIF has a robust and full process control, that acts as a quality check for bacterial trapping, bacterial lysis, DNA extraction, amplification, and probe detection; this process makes use of *Bacillus globigii*, a spore-forming soil organism [82].

The Xpert MTB/RIF assay has been found to have a limit of detection (LOD) of 131 CFU/ml of sputum [80]. The Xpert LODs for urine and other extrapulmonary specimens have not been established yet. This could have been a vital piece of information especially in the optimization phase of these extrapulmonary specimens for use in Xpert MTB/RIF studies. However the performance of Xpert MTB/RIF in sputum as reported by the World Health Organisation (WHO) is worth investigating as this gives a good forecast of how it is likely to perform in extrapulmonary specimens like urine.

Procedure: The Xpert MTB/RIF assay procedure consists of two stages that involve the manual preparation of reagents and specimen and an automated stage. The manual phase is characterized by a 15-minute treatment of the urine samples with a NaOH and isopropanol-containing reagent which decontaminates the sample thereby significantly

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eradicating any possibility of biohazard due to its ability to reduce the viability of *M. tuberculosis* [79, 81]. The urine sample: reagent buffer ratio is 1:2. The mixture is transferred manually into a disposable plastic cartridge that is preloaded with liquid reagents lyophilized reagent beads after which the cartridge is carefully inserted into the Xpert MTB/RIF assay machine. The rest of the procedures are automated [36]. Figure 2.5 summarizes all the steps and procedures of the Xpert MTB/RIF assay that should be followed. Sample extraction, amplification and detection of *M. tuberculosis* and RIF resistance is entirely automated and done in a single cartridge; and this substantially reduces cross contamination [36].

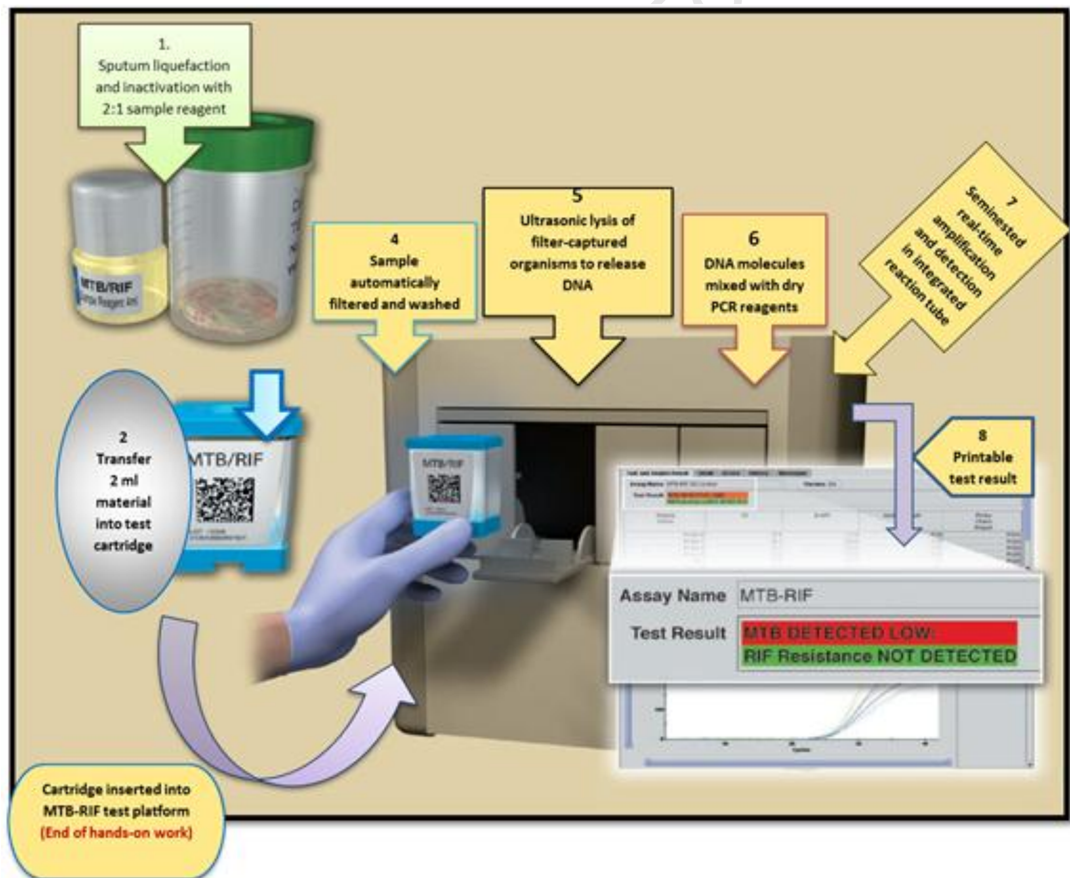

Figure 2.5. Assay Procedure for the MTB/RIF Test. 2 ml of bactericidal buffer is added to 1 ml of well-mixed urine. The resultant mixture is shaken for about 15 seconds, incubated at room temperature for 7 minutes, and shaken again and further incubated again for 8 minutes (a total of 15 minutes). 2 ml of the inactivated sample reagent: sample mixture is then taken out and transferred to the disposable plastic MTB/RIF cartridge and loaded into the GeneXpert device. The remaining steps that follow are automated. The automatically generated results are ready after 2 hours and are shown on the monitor screen reflecting whether MTB or RIF resistance have been detected [7, 8]

2.2.7.5. Xpert MTB/RIF results interpretation and evaluation
(adapted from the manufacturer's instructions)

Results were displayed in the "View Window" of the Xpert MTB/RIF as a result of fluorescent signals that are quantitated and processed through an embedded calculation algorithm in the Xpert MTB/RIF software. Final results come out as MTB NEGATIVE or MTB POSITIVE, with either RIF resistance DETECTED, RIF resistance NOT DETECTED or INDETERMINATE. In the event that the *M. tuberculosis* positive result is displays RIF resistance DETECTED, or INDETERMINATE, the amplicon would be extracted under sterile conditions on ice using an insulin syringe and transferred into a PCR appendorf tube and stored at -80°C for later sequencing. Repetition of tests were only necessary in the event of results that were displayed as "INVALID", "INDETERMINATE", "ERROR" or "NO RESULT" as these could mean that there was a problem or faulty in the way the sample preparation was done or the volume transferred into the cartridge might have been too small for processing. Cycle threshold (Ct) values were also displayed in the event of a positive result. Ct values represented a number of cycles needed for the fluorescent signal to cross a given background level or threshold. The Ct values are known to be inversely proportional to the amount of target *M. tuberculosis* DNA in the urine sample, hence a lower Ct value are representative of a higher initial concentration of the *M. tuberculosis* in the urine and higher Ct values reflect a lower initial concentration in the urine sample. Depending on the Ct value of the target *M. tuberculosis* DNA, positive results are shown as a semi-quantitation and displayed as HIGH, MEDIUM, LOW or VERY LOW as shown in table 2.2 below.

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Table 2.2. XpertMTB/RIF result name and Ct value range

MTB result	Ct range
High	<16
Medium	16-22
Low	22-28
Very Low	>28

2.2.7.6. Urine LAM ELISA assay

The LAM ELISA assay was not done in this study but the results from the previous studies [6] were available for analysis. However, a brief description of the principle and procedure of the LAM ELISA is given below.

Antibodies adsorbed on the ELISA plate capture the carbohydrate surface antigen found in positive test samples. The conjugated antibodies then attach to the captured antigen creating a sandwich assay. In the presence of the colour developer, a colour change occurs. The assay reaction is stopped using the stop solution, and the intensity of the colour (optical density) is measured using a microtiter plate reader. A positive result indicates that LAM antigen of mycobacteria is present in the sample, whereas a negative result indicates that it is not present or is below the test's detection limits (http://www.clearview.com/tb_elisa/frequently_asked_questions.aspx). The detailed procedure that was used for the LAM ELISA assay for this study has been previously described [67]. However, figure 2.5 below shows a flow diagram that summarizes the LAM ELISA procedure.

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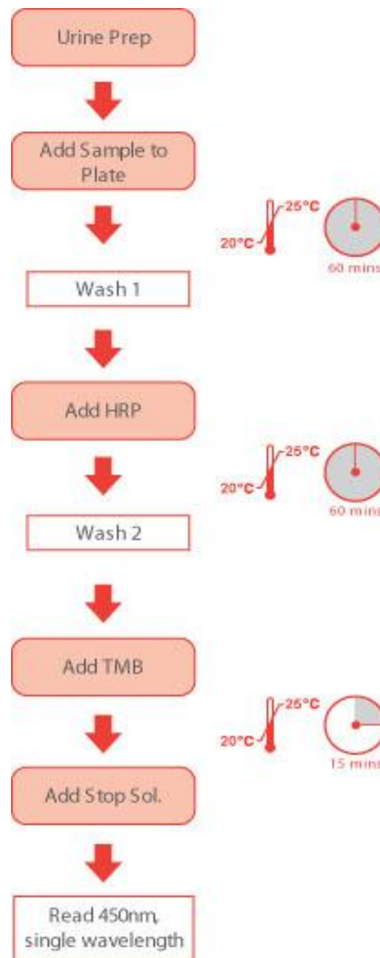

Figure 2.6. Flow diagram illustrating the steps of the Clearview TB® -LAM ELISA procedure. Urine is first boiled at 95–100°C for 30 min and centrifuged for 15 min at 10000 rpm. The supernatant is applied to the plate, an incubated for 60 min at ambient temperature, and washed with Phosphate Buffered Saline pH 7.4/Tween-20 (PBST). Subsequently, 0.1ml of undiluted conjugate solution (HRP-conjugated LAM-specific rabbit polyclonal antibody) is added. After 60 min incubation and washing with PBST, 0.1 ml of the colour developer (TMB) is administered to each well. The substrate is incubated again for 15 min at ambient temperature, and the reaction is stopped by adding 0.1 ml of stop solution (1 M H_2SO_4). The colour development is then measured at 450 nm [9].

2.2.8. Statistical analysis

A p value, $p<0.05$, was considered significant and the statistical softwares that were used to calculate these p-values were MedCalc Software Version 12.2.1 (MedCalc Software, Broekstraat 52, 9030 Mariakerke, Belgium) and XLSTAT 2012.1.02 (© Addinsoft 1995-2012). Statistical analysis was performed using GraphPad prism v5.0a (GraphPad, San Diego, CA, USA). Evaluation of the diagnostic test parameters was done using a Diagnostic or Screening Test Evaluation 1.0 using OpenEpi (Open Source

Epidemiologic Statistics for Public Health, Version 2.3. www.OpenEpi.com). Definitions for the diagnostic parameters were largely taken from MedCalc Software Version 12.2.1(MedCalc Software, Broekstraat 52, 9030 Mariakerke, Belgium).

Inter-reader agreement was analysed using Kappa statistics in the IBM SPSS Statistics 20 (SPSS Inc., 233 S. Wacker, 11th Floor, Chicago, IL 60606-6307)

2.2.8.1. Definition of diagnostic test parameters

Sensitivity: The probability that a test result will be positive when the disease is present (true positive rate).

Specificity: The probability that a test result will be negative when the disease is not present (true negative rate).

Positive predictive value (PPV): The probability that the disease is present when the test is positive.

Negative predictive value (NPV): The probability that the disease is not present when the test is negative.

2.2.8.2. Calculation of the diagnostic test parameters

The starting point for the calculation of the sensitivity, specificity, PPV and NPV was the construction of a 2x2 table with the index test results on one side of the table and the reference standard (culture) results on the other as shown in table 2.4 [86].

Table 2.4 shows the 2x2 table format that was used to calculate* the sensitivity, specificity, PPV and NPV [86]

Reference Standard			
	Disease present	Disease absent	Total
Index test positive	True positive (TP)	False positive (FP)	TP+FP
Index Test negative	False negative (FN)	True negative (TN)	TN+FN
Total	TP+FN	TN+FP	

**Calculations using the 2x2 table* [86]

Sensitivity = TP/(TP+FN)

Specificity = TN/(TN+FP)

Positive Predictive Value (PPV) = TP/(TP+FP)

Negative Predictive Value (NPV) = TN/(TN+FN)

There was no manual calculation of diagnostic accuracy parameters; all calculations were done using a statistical software called Diagnostic or Screening Test Evaluation 1.0 using OpenEpi (Open Source Epidemiologic Statistics for Public Health, Version 2.3. www.OpenEpi.com).

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CHAPTER 3: GROUP 1 RESULTS AND DISCUSSIONS

In this chapter, the diagnostic accuracy and inter-rater agreement results for the urine LAM strip test in group 1 ambulatory patients is presented.

3.1. Group 1 patient characteristics

Figure 3.1 provides a study outline of *M. tuberculosis* culture and HIV status, together with urine LAM strip test results. 300 participants were enrolled for this study and one participant was unable to provide a urine sample at the time of enrolment. 96% (287/299) had *M. tuberculosis* culture results. 27% (80/291) were HIV positive. Amongst the HIV positive cases, 63% (50/80) had CD4 ≤200 cells/ml, and 9% (7/80) had unknown CD4 count at the time of study. The characteristics and demographics of this study are shown in table 3.1. The median (IQR) age of patients was 39 (IQR: 31-46); and the majority of patients were males (63%) and of African race (97%). A greater proportion of HIV-infected compared to un-infected patients had a history of TB [54% (95% CI: 43-65) vs. 40% (95% CI: 34-47), p=0.04]. Also, there was a significantly larger proportion of males uninfected with HIV than those infected [72% (95% CI: 66-78) vs. 40% (95% CI: 30-51), p<0.001]. Moreover, there were comparable proportions of *M. tuberculosis* culture positive and culture negative patients with a history of TB [45% (95% CI: 30-61) vs. 43% (95% CI: 37-49); p=0.006, respectively]. Among patients who reported weight loss, 83% (95%CI: 68-92) had *M. tuberculosis* culture-confirmed TB compared to 57% (95%CI: 51-63) who were *M. tuberculosis* culture negative, p=0.002. A significantly higher proportion of patients with *M. tuberculosis* culture positive compared to culture negative had reported having fever (>38°C) [13% (95% CI: 5-26) vs. 2% (95% CI: 1-4), p=0.007]. More so, there were more HIV infected patients than uninfected among the same patients reporting

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fevers [8% (95% CI: 3-15) vs. 2% (95% CI: 1-5), p=0.03]. There were more patients with a CD4 >200 cells/ml compared to patients with CD4 ≤200 cells/ml in patients who experienced night sweats [87% (95% CI: 68-98) vs. 50% (95% CI: 36-64), p<0.002].

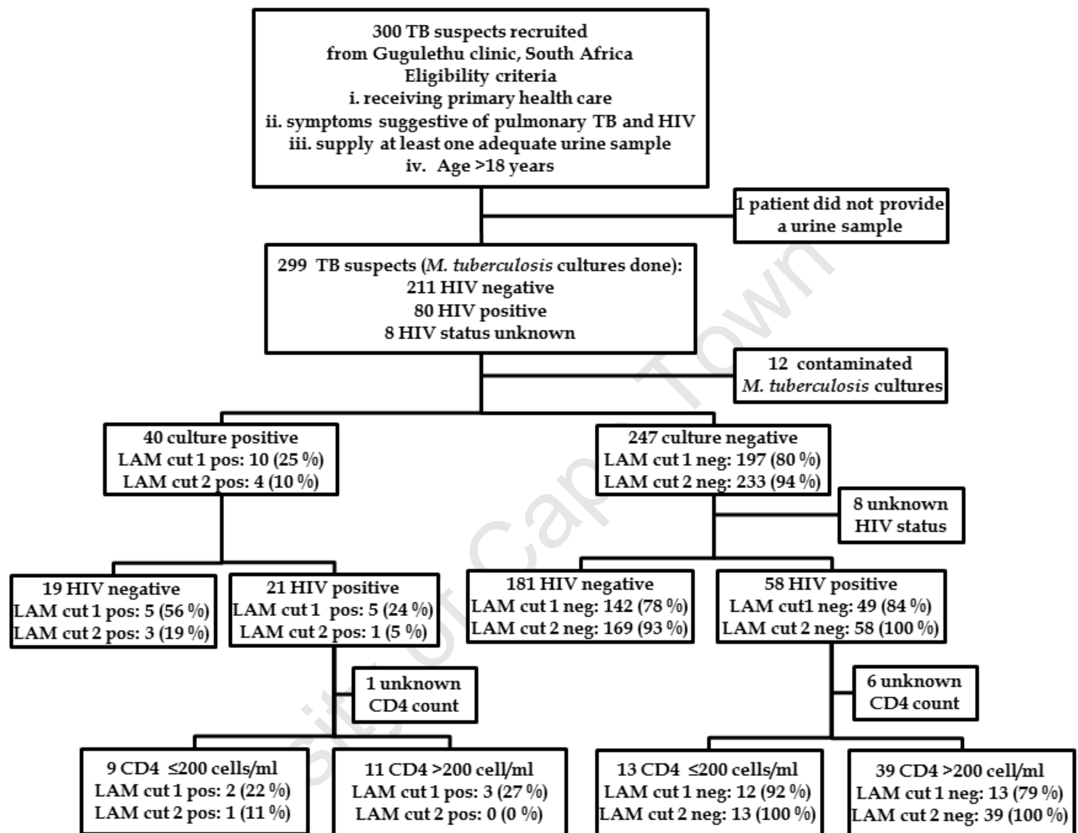

Figure 3.1. Study flowchart showing TB diagnostic grouping, HIV status, CD4 count grouping and LAM cut-1 and LAM cut-2 results for each category.

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Table 3.1. The characteristics and demographic features of the study patients stratified according to *M. tuberculosis* culture results, HIV status and CD4 cell count.

Demographics	All (n=299)	[#]*M. tuberculosis* culture positive (n=40)	[#]*M. tuberculosis* culture negative (n=247)	[@]HIV negative (n=211)	[@]HIV positive		
					All (n=80)	*CD4 ≤200 cells/ml(n=50)	*CD4 >200 cells/ml (n=23)
Median age (years) (IQR)	39 (31-46)	36 (30-47)	36 (28-44)	40 (35-47)	36 (30-42)	37 (29-43)	32 (30-41)
Male sex	188 (63)	25 (63)	153 (62)	[Θ]152 (72)	[Θ]32 (40)	16 (32)	10 (44)
Race Black	290 (97)	39 (98)	239 (97)	205 (97)	77 (96)	47 (94)	23 (100)
Coloured	8 (3)	1 (3)	7 (3)	5 (2)	3 (4)	3 (6)	0 (0)
Other	1 (0.3)	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Previous TB	128 (43)	[δ]18 (45)	[δ]105 (43)	[¶]85 (40)	[¶]43 (54)	28 (56)	10 (44)

Clinical features

Cough >2wks	264 (88)	37 (92)	216 (87)	189 (90)	67 83)	42 (84)	19 (83)
Night sweats	147 (49)	33 (83)	194 (79)	168 (80)	65 (82)	[Φ]25 (50)	[Φ]20 (87)
Weight loss	181 (61)	[¥]33 (83)	[¥]141 (57)	126 (60)	54 (68)	33 (66)	15 (65)
Fever >38°C	9 (3)	[€]5 (13)	[€]5 (2)	[³]4 (2)	[³] 6 (8)	2 (4)	3 (13)

#There were 12 contaminated *M. tuberculosis* cultures; [@]8 participants had unknown HIV results; *7 participants had unknown CD4 count results

[δ]p=0.006, [¥]p=0.002, [€]p=0.007, [³]p=0.03, [¶]p=0.04, [Θ]p<0.001, [Φ]p=0.002; p>0.05 not shown in table.

3.2. Inter-reader agreement

Table 3.2 provides the overall grading agreement for the urine LAM strip test. Proportionate agreement was 82% (244/299) with a kappa of 0.52, which was moderate.

Table 3.2. Cross-tabulation of urine LAM strip grading between two independent readers

***Reader 1 and 2 crosstabulation**							
Count							
Grade		reader1					Total
		0	**1**	**2**	**3**	**4**	
reader2	**0**	204	17	0	0	0	221
	1	30	34	3	1	0	68
	2	0	0	6	3	0	9
	3	0	0	0	0	1	1
Total		234	51	9	4	1	299

*Measure of Agreement, Kappa=**0.518**

Table 3.3 shows the cross-tabulation of the urine LAM strip test results for 2 readers with results dichotomized at using the manufacturer's suggested grade-1 cut-point i.e. grade 1-5 considered test positive and only grade 0 considered test negative. The proportionate agreement was 84.3% (95%CI: 80.1-88.4) (252/299) with a kappa value of 0.57 which was moderate.

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Table 3.3. Cross-tabulation of urine LAM strip grading between two independent readers using dichotomized results at the manufacturer's recommended grade-1cut-point *(Grade0=0; Grade 1-5=1)*

*Reader 1 and 2 crosstabulation				
Count				
		Reader 1		Total
		Neg	Pos	
Reader 2	Neg	204	17	221
	Pos	30	48	78
Total		234	65	299

*Measure of Agreement, Kappa = **0.57**

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Table 3.4 shows the cross-tabulation of the urine LAM strip test results for 2 readers with results dichotomized at using the alternative grade-2 cut-point i.e. grade 2-5 considered test positive and grade 0-1 considered test negative.

Table 3.4. The inter-reader agreement between the two LAM strip readers using our grade-2 cut-point.

#Reader2 and reader1 crosstabulation				
Count				
		Reader2		Total
		Neg	Pos	
Reader1	Neg	285	0	285
	Pos	4	10	14
Total		289	10	299

#Measure of Agreement, Kappa = **0.83**

The proportionate agreement between the two readers using a grade-2 cut-point was 98.7% (95%CI: 97.3-100) (295/299) with a kappa of 0.83. Proportionate agreement was higher using the alternative grade-2 cut-point compared with the manufacturer's suggested grade-1 cut-point [LAM cut-2:

98.6% (95%CI: 97.3-100) 295/299 vs. LAM cut-1: 84.3% (95%CI: 80.1-88.4) 252/299 vs; $p<0.001$].

3.3. Inter-reader agreement in HIV-infected patients

Table 3.5 shows that in HIV-infected patients, the proportionate agreement between the two readers using a grade-1 cut-point was 84.7% (95%CI: 78.2-88.1) (180/215) with a kappa value of 0.58 which was moderate.

Table 3.5. Cross-tabulation of urine LAM strip grading between two independent readers using dichotomized results at the grade-1 cut-point among the HIV negative subjects.

*Reader1 and reader2 Crosstabulation				
Count				
		reader2		Total
		Neg	Pos	
reader1	Neg	142	23	165
	Pos	12	38	50
Total		154	61	215

*Measure of agreement, Kappa = **0.58**

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Table 3.6, shows that the overall proportionate agreement between the two readers in the HIV negative patients, using grade-2 cut-point, was 98.6% (95%CI: 96.0-99.5) (212/215) with a kappa value of 0.85 which was almost perfect.

Table 3.6. Cross-tabulation of urine LAM strip grading between two independent readers using dichotomized results at the grade-2 cut-point among the HIV negative subjects.

*Reader1 and reader2 crosstabulation				
Count				
		reader2		Total
		Neg	Pos	
reader1	Neg	203	0	203
	Pos	3	9	12
Total		206	9	215

*Measure of agreement, Kappa = **0.85**

The overall proportionate agreement between the two readers in the HIV positive patients, using grade-1 cut-point, was 84.2% (95%CI: 74.4-90.7) (64/76) with a kappa value of 0.44 which was moderate, as shown in table 3.7 below.

Table 3.7. Cross-tabulation of urine LAM strip grading between two independent readers using dichotomized results at the grade-1 cut-point among the HIV positive subjects.

*Reader1 and reader2 crosstabulation				
Count				
		reader2		Total
		Neg	Pos	
reader1	Neg	57	7	64
	Pos	5	7	12
Total		62	14	76

*Measure of agreement, Kappa = **0.44**

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In table 3.8 below, the overall measure of inter-reader agreement between the two readers in the HIV negative patients, using cut-point 2 was 100% (95%CI: 95.2-100) (180/215) with a kappa value of 1.00 which, was a perfect agreement.

Table 3.8. Cross-tabulation of urine LAM strip grading between two independent readers using dichotomized results at the grade-2 cut-point among the HIV positive subjects.

*Reader1 and reader2 crosstabulation				
Count				
		reader2		Total
		Neg	Pos	
reader1	Neg	75	0	75
	Pos	0	1	1
Total		75	1	76

*Measure of agreement, Kappa = **1.00**

Table 3.9, below, shows that there was a significant difference in the inter-reader agreement using of cut-point 1 versus cut-point 2 in both HIV positive and negative patients. However, there was no significant difference between cut-point 1 and cut-point 2 within each HIV group.

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Table 3.9. Different Kappa values and the proportion of the overall agreement between the two readers for each HIV infected and non-infected groups of patients using grade-1 and grade-2 cut-points.

HIV status	Kappa values		Overall proportional agreement % (95% CI)		
	Cut-point 1	Cut-point 2	Cut-point 1	Cut-point 2	P-value
Negative (n=215)	0.58	0.85	83.7 (78.2-88.1) #180/215	98.6 (96.0-99.5) *212/215	<0.0001
Positive (n=76)	0.44	1.00	84.2 (74.4-90.7) #64/76	100 (95.2-100) *76/76	0.0009
***Overall (N=299)**	0.57	0.83	84.3% (80.1-88.4) 252/299	98.6% (97.3-100) 295/299	<0.001

*8/299 patients had no HIV results (either refused or unknown).

and * refer to the comparison of proportional agreement between HIV negative and positive patients within each cut-point; #p=0.94; * p>0.99

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3.4. Urine LAM strip test diagnostic accuracy

Table 3.10 shows diagnostic accuracy measures for the urine LAM strip test stratified by cut-point. Overall sensitivity, irrespective of cut-point was poor. Sensitivity was 25% (95%CI: 14-40, 10/40) and 10% (95%CI: 4-23, 4/40) for the grade-2 and grade-1 cut-points respectively (p=0.09). By contrast, test specificity significantly increased from 79.8% (95%CI: 74.3-84.3, 197/247) when using the grade-1 to 94.3% (95% CI: 91.0-96.6, 233/247) when using the grade-2 cut-point (p<0.001). At a disease prevalence of 13%, the use of the grade-2 vs. grade-1 cut-point did not increase PPV [16.7% (95%CI: 9.3-28.0, 10/60) vs 22.2% (95%CI: 9.0-45.2, 4/18), p=0.647]. Table 3.10 shows the summary of diagnostic performance of the LAM strip test using different cut-points.

Table 3.10. Diagnostic accuracy of the urine LAM strip test using the manufacturer's grade-1 and the alternative grade-2 cut-points.

Parameter	Overall performance (N=287)		
	Cut-point 1	**Cut-point 2**	***P-value**
Sensitivity (%)	25	10%	0.09
(95%CI)	(14.2-40.2)	(4.0-23.1)	
n	10/40	4/40	
Specificity (%)	79.8%	94.3%	<0.001
(95%CI)	(74.3-84.3)	(91.0-96.6)	
n	197/247	233/247	
PPV (%)	16.7%	22.2%	0.59
(95%CI)	(9.3-28.0)	(9.0-45.2)	
n	10/60	4/18	
NPV (%)	86.8%	86.6%	0.96
(95%CI)	(81.8-90.6)	(82.0-90.2)	
n	197/227	233/269	

*Indicates p-values for a comparison of the LAM strip overall performance between grade-1 and grade-2 cut-points.

3.4.1. Urine LAM strip test diagnostic accuracy in HIV infected patients

Table 3.11 shows diagnostic accuracy measures for the urine LAM strip test stratified by HIV status and grade cut-point. In HIV positive patients sensitivity did not decrease significantly when using the grade-2 vs. grade-1 cut-points [5.3% (95%CI: 0.9-24.6; 1/19) vs. 21.1% (95%CI: 8.5-43.3; 4/19), p=0.19. However, in HIV positive patients specificity was significantly higher when using the grade-2 compared to the grade-1 cut-point [100% (95%CI: 93.8-100; 58/580) vs 84.48 (95%CI: 73.0-91.6; 49/58); p=0.009]. More so, specificity was significantly superior in HIV positive patients than HIV

negative patients [100% (95%CI: 93.8-100; 58/58 vs 93.4% (95%CI: 88.8-96.1; 169/181); p=0.02]. No changes were seen in the NPVs in both HIV positive and HIV negative patients when a grade-2 cut-point was used, though there was a significant difference in the NPVs between the HIV positive and negative patients within each cut-point.

Table 3.11. Diagnostic accuracy of the urine LAM strip test using the manufacturer's grade-1 and the alternative grade-2 cut-points stratified by HIV status.

Test Performance	Grade-1 cut-point			Grade-2 cut-point		
	HIV positive (n=74)	**HIV negative (n=200)**	**P-value**	**HIV positive (n=74)**	**HIV negative (n=200)**	**P-value**
Sensitivity% (95% CI) n	23.81 (10.6-45.1) 5/21	55.6 (26.7-81.1) 5/9	0.12	4.8 (0.8-22.7) 1/21	18.8 (6.6-43.0) 3/16	0.23
Specificity% (95% CI) n	*84.48 (73.0-91.6) 49/58	#78.5 (71.9-83.8) 142/181	0.32	*100 (93.8-100) 58/58	#93.4 (88.8-96.1) 169/181	0.02
PPV (95% CI) n	35.71 (16.3-61.2) 5/14	11.4 (5.0-24.0) 5/44	0.06	100 (20.7-100) 1/1	20 (7.0-45.2) 3/15	0.06
NPV% (95% CI) n	75.38 (63.7-84.2) 49/65	97.3 (93.2-98.9) 142/146	<0.001	74.4 (63.7-82.7) 58/78	92.9 (88.2-95.8) 169/182	<0.001

*p=0.009; #p=0.0001; p>0.05 for comparisons between HIV positive or negative grade-1 cut-point and HIV positive or negative cut-point 2 not indicated.
P<0.05 for comparisons between HIV positive and HIV negative patients within each cut-point are highlighted in blue.

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3.4.2. Conclusion

The urine LAM strip test, when used among ambulant TB suspects presenting at a primary health care facility, has poor overall sensitivity,

similar to that of the preceding urine LAM ELISA, even among HIV-coinfected patients. The use of a grade-2 rather than the manufacturer's suggested grade-1 cut-point optimises rule-in test utility by improved test specificity, and furthermore the grade-2 cut-point greatly improved inter-reader reliability.

These results confirm the findings of Peter et al. (2012), and support the preferred use of the grade-2 rather than the grade-1 cut-point [6]. Furthermore, the overall poor sensitivity of the LAM strip test in this study is similar to that of previous studies that were carried among out-patients, as shown in table 1.4, using the preceding urine LAM ELISA. For instance, the LAM ELISA sensitivity was 5.8% in one study [87]. In addition, Dheda *et al* (2009) produced an overall sensitivity of 13% using LAM ELISA and a specificity of 99%, which is comparable to our LAM strip grade-2 cut-point sensitivity and specificity of 10% and 94% respectively [67].

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CHAPTER 4: GROUP 2 PATIENT RESULTS AND DISCUSSION

This chapter presents the diagnostic accuracy of urine-based LAM and MTB/RIF in a cohort of hospitalised TB HIV co-infected patients with advanced immunosuppression. We have examined the sensitivity of urine-based tests alone or in combination for smear-negative or sputum scarce TB.

4.1. Study population

The study population involved a consecutive series of well-defined 116 *M. tuberculosis* culture positive HIV-TB co-infected patients. These patients were at least 18 years of age and had illnesses requiring hospitalization; they were recruited from 4 hospitals in Cape Town. 3/116 patients were missing urine samples. 54/113 (48%) were either sputum smear-negative or sputum scarce (SN/SS) [20/54 (37%) sputum scarce and 34/54 (63%) sputum negative]. Figure 4.1 shows the study plan and the important urine diagnostic test results.

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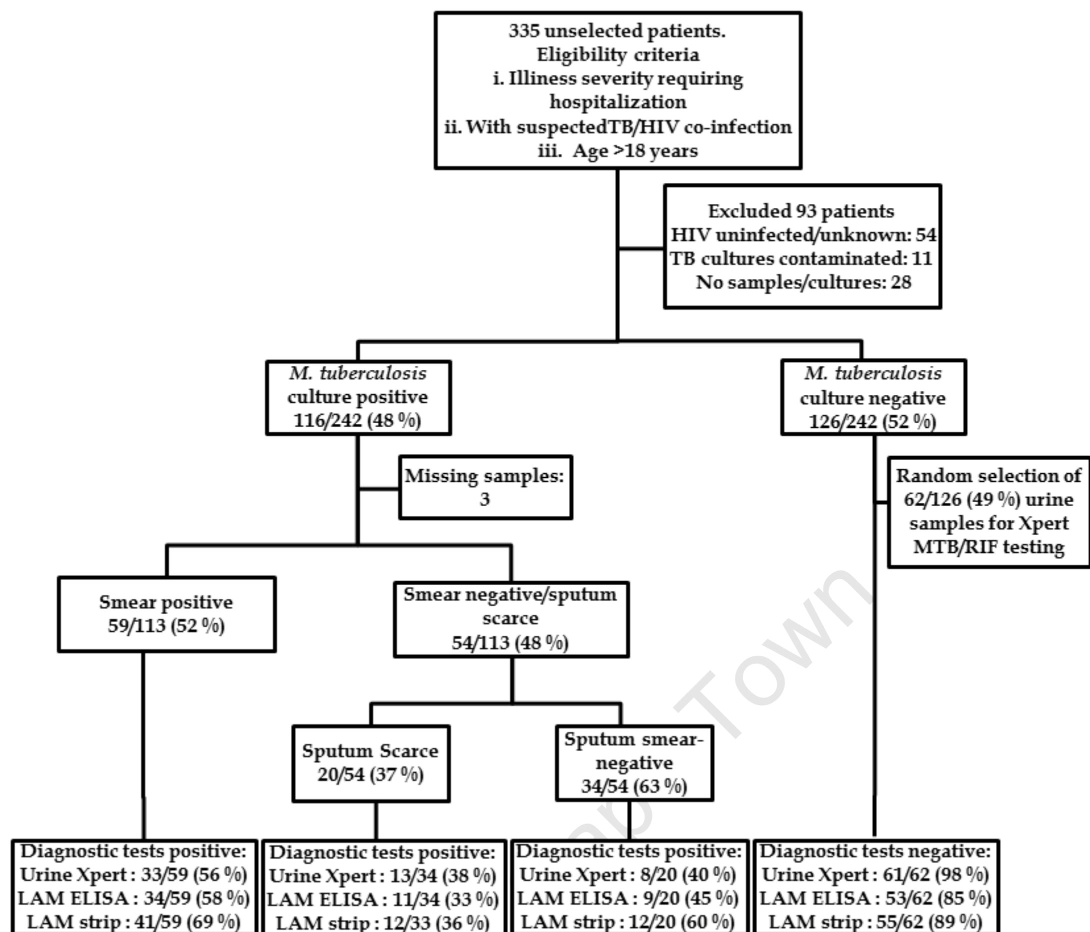

Figure 4.1. Summary of the study population groups and the diagnostic performance of Urine LAM ELISA, urine LAM strip test and urine Xpert MTB/RIF in each the patient group.

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4.2 The demographic and clinical characteristics of study patients

The patient demographic and clinical features of the study group stratified by sputum smear status, CD4 cell count and Xpert MTB/RIF results are shown in Table 4.1. The median (interquartile range) age was 35 (28-38) years and the cohort was predominantly females (59%). In HIV-infected patients, the median (IQR) CD4 cell count was 89 (45-195) cells/ml and 75% of patients were on ARVs at enrolment. 32/113 (28%) had previous TB and 25/113 (22%) were current smokers. The commonest symptom was self-reported weight loss (90%).

Table 4.1. Demographic, clinical and microbiological characteristics of *M. tuberculosis* culture positive and HIV-infected patients in the study population stratified by CD4 cell count, sputum smear status and urine-based MTB/RIF result†.

Patient characteristic(s)	At least 1 sputum or non-sputum *M. tuberculosis* culture positive (N=116) n (%)	Sputum smear positive (N=60) n (%)	Sputum smear-negative or sputum scarce (N=56) n (%)	CD4 ≤200 cells/ml# (N=81) n (%)	CD4 >200 cells/ml# (N=26) n (%)	Urine Xpert MTB/RIF negative, *M. tuberculosis* culture positive (N=59) n (%)
Age (years) (median, IQR)	35 (28-39)	35 (28-39)	35 (28-39)	35 (29-39)	36 (27-38)	35 (28-38)
Male	47(41)	22 (37)	25 (45)	34 (42)	7 (27)	19 (32)
CD4 cell count (median, IQR)	86 (42-192)	108 (50-215)	75 (33-160)	59 (34-110)	318 (222-497)	142 (59-241)
Previous TB	33 (29)	19 (32)	14 (25)	21 (26)	8 (31)	19 (32)
Current Smoker	25 (22)	15 (25)	10 (18)	16 (20)	6 (23)	12 (20)
Clinical features						
Cough >2wks	99 (85)	54 (90)	45 (80)	68 (84)	21 (81)	50 (85)
Night sweats	82 (71)	46 (77)	36 (64)	57 (70)	18 (69)	39 (66)
Weight loss	105 (91)	53 (88)	52 (93)	74 (91)	22 (85)	50 (85)
Fever >38°C	29 (25)	19 (32)	10 (18)	21 (26)	5 (19)	13 (22)

†no significant differences between patient groups were noted for any of the patient characteristics; #9 patients did not have data CD4 count data

4.3. Diagnostic accuracy of sputum smear microscopy and urine-based diagnostics

Table 4.2 shows the sensitivity and specificity of sputum-based smear microscopy compared to urine-based LAM ELISA, strip test and MTB/RIF. When used alone, all included tests offered equivalent overall sensitivity, between 48-58%. . Among the urine-based diagnostic methods, MTB/RIF had a significantly higher specificity compared with LAM ELISA and LAM strip tests [Urine MTB/RIF: 98%, (95%CI: 95-100, 61/62) vs. LAM ELISA: 89%, (95%CI: 81-97, 55/62); p=0.03 and LAM strip test: 85%, 95%CI: (77-94), 53/62; p=0.009]. When used in combination, the sensitivity of urinary LAM ELISA with sputum smear microscopy was significantly higher than either test alone [74%, 95%CI: (65-82), 83/113 vs LAM ELISA: 58%, (95%CI: 49-67, 65/112); p=0.01 or Smear microscopy: 52%, 95%CI: (43-61), 59/113, p=0.001]. The sensitivity of urinary LAM ELISA combined with MTB/RIF was significantly higher than MTB/RIF or LAM strip testing alone [68%, 95%CI: (60-77), 77/113 vs MTB/RIF : 48%, 95%CI: (39-57), 54/113; p=0.002 or LAM strip:48%, 95%CI: (39-57), 55/113; p=0.003]. Nevertheless, the combined urinary LAM ELISA and MTB/RIF was not significantly higher than LAM ELISA alone [68%, 95%CI: (60-77), 77/113; vs 58%, (95%CI: 49-67, 65/112); p=0.1 respectively].

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Table 4.2. Diagnostic accuracy of sputum smear microscopy, urinary MTB/RIF, TB LAM ELISA, LAM strip test (grade-2 cut-point) and their clinically relevant combinations

Diagnostic test(s)	All *M. tuberculosis* culture positive (N=113)	Random sample of *M. tuberculosis* culture negative patients (N=62)
	Sensitivity (%) (95% CI)	**Specificity (%) (95% CI)**
Sputum smear microscopy	52[#3] (43-61) 59/113	100 (94-100) 62/62
Urine MTB/RIF	48[#1] (39-57) 54/113	98[#5#6] (95-100) 61/62
Urine LAM ELISA	58[#4] (49-67) 65/112	89[#5] (81-97) 55/62
Urine LAM strip test (grade-2 cut-point)	48[#2] (39-57) 55/113	85[#5] (77-94) 53/62
Urine LAM ELISA followed by urine MTB/RIF (performed if LAM ELISA negative)	68[#1 #2] (60-77) 77/113	89 (81-97) 55/62
Urine LAM ELISA combined with smear microscopy	74[#3 #4] (65-82) 83/113	89 (81-97) 55/62

[#]Indicates p<0.05 for a comparison of the sensitivity between different tests (e.g. urine MTB/RIF vs. LAM strip test) or combinations thereof; specific p-value: [#1]p=0.002; [#2]p=0.003; [#3]p=0.001; [#4]p=0.01;; [#5]p=0.03; [#6]p=0.009 non-significant p-values not shown.

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4.4. Diagnostic accuracy of sputum smear microscopy and urine-based diagnostics stratified by CD4 cell count

Urine-based MTB/RIF sensitivity in patients with CD4≤200 cells/ml was significantly higher [54%, 95%CI: (43-65), 42/78] compared to [31%, 95%CI: (17-50), 8/26] for CD4>200 cells/ml (p=0.05). Urinary LAM ELISA and strip tests also had higher sensitivities in patients with CD4<200 cells/ml (table 4.3). All urine-based diagnostics had shown the highest sensitivities in patients with CD4<100 cells/ml, but sensitivities were not significantly

different to patients with CD4<200 cells/ml. In contrast, the sensitivity of sputum smear microscopy was unaffected by decreasing CD4 cell count.

Table 4.3. Sensitivities of sputum smear microscopy, urinary MTB/RIF, TB LAM ELISA, LAM strip test (grade-2 cut-point) and their clinically relevant combinations in any sputum/non-sputum *M. tuberculosis* culture positive patients stratified by CD4 cell count[†]

Diagnostic test(s)	CD4 count >200 cells/ml (N=26)	CD4 count ≤200 cells/ml (N=78)	CD4 count ≤100 cells/ml (N=54)	P-value
	Sensitivity (%) (95% CI)	Sensitivity (%) (95% CI)	Sensitivity (95% CI)	
Sputum smear microscopy	58[#1 #2#6] (39-75) 15/26	50[#3] (39-61) 39/78	57[#9] (42-70) 25/54	[*]0.5
Urine MTB/RIF	31[#2 @4] (17-50) 8/26	54[#4#7] (43-65) 42/78	61[#11 @4] (48-73) 33/54	[*]0.04
Urine LAM ELISA	27[#6 @3] (14-46) 7/26	69[#3] (58-78) 53/77	74[@3] (60-84) 39/53	[*]0.001
Urine LAM strip test (grade-2 cut-point)	27[#1 @2] (14-46) 7/26	56[#5#8] (45-67) 44/78	63[#10 @2] (50-75) 34/54	[*]0.009
Urine LAM ELISA followed by urine MTB/RIF (if LAM ELISA negative)	38[@1] (20-57) 10/26	79[#4 #5] (71-88) 62/78	87[#10#11 @1] (76-94) 47/54	[*]0.001
Urine LAM ELISA combined with smear microscopy	58 (39-77) 15/26	80[#7#8] (71-88) 62/78	78[#9] (65-87) 42/54	[*]0.03

[#]indicates p<0.05 for a comparison of the sensitivity between different tests or combinations thereof; specific p-value: [#1]p=0.02; [#2]p=0.05; [#3]p=0.02; [#4]p<0.001; [#5]p=0.002; [#6]p=0.03; [#7]p=0.001; [#8]p=0.02; [#9]p<0.001; [#10]p=0.004, [#11]p=0.002; [#12]p<0.001
[*]p-values for comparisons between CD4 count >200 cells/ml and CD4 count ≤200 cells/ml
[@]p-values for comparisons between CD4 count >200 cells/ml and CD4 count ≤100 cells/ml; [@1]p<0.001; [@2]p=0.003; [@3]p<0.001; [@4]p<0.01
[†]9 culture positive patients with no available CD4 cell count results

When urine-based diagnostics were used in combination or together with sputum smear microscopy, the highest overall sensitivity was in patients with a CD4<100 cells/ml using a combination of urine LAM ELISA and MTB/RIF [87% (95%CI (76-94)]. When compared to urine or sputum

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diagnostic tests alone, test combinations performed similarly in different CD4 cell count strata to in the overall group (see Table 4.2 and 4.3).

4.5. Diagnostic accuracy of sputum smear microscopy and urine-based diagnostics stratified by site of disease

The sensitivities (95% CI) of smear microscopy, urinary MTB/RIF, LAM ELISA and strip test stratified by sites of *M. tuberculosis* culture positivity are shown in table 4.4.

Table 4.4. Sensitivities of Sputum smear, GeneXpert, LAM ELISA, LAM strip test (grade-2 cut-point) and their clinically relevant combinations stratified with site of disease in *M. tuberculosis* culture confirmed TB/HIV coinfected patients.

	Site of disease		
	Pulmonary-TB only (N=68)	**Pulmonary- and extrapulmonary - TB (N=19)**	**Extrapulmonary-TB only (N=26)**
Diagnostic test(s)	**Sensitivity % (95% CI)**	**Sensitivity % (95% CI)**	**Sensitivity % (95% CI)**
Sputum smear	66 (54-76) 45/68	74 (51-88) 14/19	N/A
LAM ELISA*	[#1]58 (46-69) 39/67	68 (46-85) 13/19	50 (32-68) 13/26
LAM strip test (grade-2 cut-point)	[#2]50 (38-62) 34/68	58 (36-77) 11/19	[#5]39 (22-57) 10/26
Urine Xpert MTB/RIF	[#3#4]44 (33-56) 30/68	63 (41-81) 12/19	46 (29-65) 12/26
Xpert and ELISA	[#4]65 (53-75) 44/68	84 (62-94) 16/19	[#5]69 (50-83) 18/26
LAM ELISA and sputum smear	[#1#2#3]77 (65-85) 52/68	84 (62-95) 16/19	N/A

*There was a missing LAM ELISA result

P-values ($p<0.05$) of differences between combined diagnostic tests and individual tests, [#1]p=0.03; [#2]p=0.001; [#3]p=0.0001; [#4]p=0.02; [#5]p=0.03; p>0.05 not shown.

Table 4.4 shows that all the tests perform better in patients that had both PTB and EPTB than the patients that were presented with PTB only or EPTB only; however there was lack of significant difference between each of these groups. Combined LAM ELISA and urine Xpert MTB/RIF had significantly better sensitivity than individual Urine Xpert MTB/RIF [65%, 95%CI: (53-75), 44/68 vs 44%, 95%CI: (33-66), 30/68; p=0.02] in the PTB patients; more so the same diagnostic combination was significantly superior to individual LAM strip test (grade-2 cut-point) in patients with EPTB only [69%, 95%CI: (50-83), 18/26 vs 39%, (95%CI: (22-57), 10/26; p=0.03]. In patients presenting with both PTB and EPTB, both combined diagnostic tests had similar sensitivities and were better than the individual test, notwithstanding lack of statistical significance between the combined tests and the individual tests.

4.6. Diagnostic accuracy of urine-based diagnostics stratified by smear status

Table 4.5 shows the sensitivity and specificity of urinary Xpert MTB/RIF, TB LAM ELISA, LAM strip test (grade-2 cut-point) and different combinations thereof in 59 sputum smear positive and 54 sputum smear-negative/sputum scarce patients. In SN/SS patients the sensitivities of urinary MTB/RIF, LAM ELISA and LAM strip test were 39%, 45%, and 3% respectively. A combination of urine MTB/RIF and ELISA had the highest overall sensitivity [60% (95% CI: 47-72)], which was better than urine MTB/RIF and LAM strip alone (60% vs 39%, p=0.03, and 37%, p=0.02 respectively), but not LAM ELISA alone (60% vs 45%, p=0.12).

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Table 4.5. The sensitivity of urinary Xpert MTB/RIF, TB LAM ELISA, LAM strip test (grade-2 cut-point) and combinations thereof, stratified by smear status and ability to produce a sputum sample (sputum scarce).

Diagnostic test(s)	Smear negative/sputum scarce (n=54)	Sputum positive (n=59)	[@]P-value
	Sensitivity (95% CI	Sensitivity (95% CI	
LAM ELISA*	45 (33-59) 24/53	69 (57-80) 41/59	**0.01**
LAM strip test (grade-2 cut-point)	[#1#3]37 (25-50) 20/54	[#5]59 (47-71) 35/59	**0.02**
Urine Xpert MTB/RIF	[#2]39 (27-52) 21/54	[#4]56 (43-69) 33/59	0.07
Xpert and ELISA	[#2#3]60 (47-72) 32/53	[#4#5]76 (64-85) 45/59	0.08
Xpert and LAM strip test (grade-2 cut-point)	[#1]57 (44-70) 31/54	69 (57-80) 41/59	0.2

#indicates $p<0.05$ for a comparison of the sensitivity between different tests (e.g. urine MTB/RIF vs. LAM ELISA) or combinations thereof within each group; specific p-value: [#1]p=0.04; [#2]p=0.03; [#3]p=0.02; [#4]p=0.02; [#5]p=0.05; p>0.05

@ indicates p-values for comparison of sensitivity of SS/SN vs sputum positive; $p<0.05$ are highlighted in blue.

*There was a patient who had no LAM ELISA result.

These urine based tests had higher sensitivities in the SP patients than in the SS/SN patients as shown in table 4.5.

4.6.1. Diagnostic accuracy of urine-based diagnostics in sputum scarce (SS) and sputum smear-negative (SN) patients

The sensitivity of urinary Xpert MTB/RIF, TB LAM ELISA and LAM strip test (grade-2 cut-point) in study patients stratified according to their ability to produce sputum and smear negativity is outlined in table 4.6. LAM ELISA had a superior sensitivity [60%, 95%CI: (39-78), 12/20] in patients who could not produce sputum compared to both urinary Xpert MTB/RIF and LAM strip test (grade-2 cut-point), notwithstanding there was no statistical significance. Also, urine LAM ELISA performance in the SS patients was higher than that of the SN group [60% (39-78), 12/20 vs. 36% (22-53), 12/33 (p=0.1)]. There was generally a poor performance throughout all diagnostic tests within the sputum smear-negative patients. Xpert MTB/RIF had a sensitivity of 38%, (95%CI: 24-55, 13/34) which was equivalent to urine LAM ELISA and LAM strip test.

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Table 4.6. Sensitivity of urinary Xpert MTB/RIF, TB LAM ELISA, LAM strip test (grade-2 cut-point) and combinations thereof, stratified by smear status and ability to produce a sputum sample (sputum scarce).

	Sputum scarce (n=20)	Smear negative (n=34)	
Diagnostic test(s)	**Sensitivity (95% CI**	**Sensitivity (95% CI**	#P-value
LAM ELISA*	60 (39-78) 12/20	36 (22-53) 12/33	0.1
LAM strip test (grade-2 cut-point)	45 (26-66) 9/20	*[1]32 (19-49) 11/34	0.4
GeneXpert	40 (22-61) 8/20	38 (24-55) 13/34	0.5
Xpert and ELISA*	70 (48-85) 14/20	55 (38-70) 18/33	0.2
Xpert and LAM strip test (grade-2 cut-point)	55 (34-74) 11/20	*[1]59 (42-74) 20/34	0.7

# indicates p-values for a comparison of the sensitivity between different tests (e.g. urine MTB/RIF vs. LAM ELISA) or combinations thereof between each patient group (i.e. SS and SN).
*denotes p<0.05 for a comparison of differences in sensitivity within each group for a specific test or combinations thereof; *[1]p=0.03

There was an increase in the test performance when diagnostic tests were combined**.** Combined Xpert MTB/RIF and LAM strip test (grade-2 cut-point) had equivalent moderate sensitivities in both the SS and SN patients. There was, however, a significant difference, in the SN patients, between the combined Xpert MTB/RIF and LAM strip test (grade-2 cut-point) [59% 95%CI: (42-74), 20/34 vs 32%, 95%CI: (19-49), 11/34 (p=0.03)-LAM strip test alone].

4.7. The effect of urine centrifugation on urine MTB/RIF performance

In 33% (38/116) of *M. tuberculosis* culture positive patients there was sufficient urine available to perform urine MTB/RIF using both 1ml of

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unprocessed urine and 2-10 mls of centrifuged and pelleted urine. The median (IQR) of urine used for pelleting was 10 (5-10) ml. The sensitivity of urine Xpert MTB/RIF was higher using 2-10ml centrifuged and pelleted vs. 1ml unprocessed urine [42% (95%CI: 26-58; 16/38) vs. 8% (95%CI: 0-16; 3/38), $p<0.001$]. There was no significant increase in sensitivity when 6-10mls of urine were centrifuged and pelleted compared with 2-5mls [48% (95%CI: 29-67; 13/27) vs. 28% (95%CI: 0-54; 3/11), $p=0.2$].

4.8. Conclusion and brief discussion (a further more detailed discussion is presented at the end of the thesis)

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The main findings of this study show that performing urine MTB/RIF was possible and offers potential utility for non-sputum based diagnostic, especially for sputum scarce and sputum smear-negative patients, the majority of whom had EPTB. In this patient group urine MTB/RIF detected close to half of all TB cases. Furthermore, concentrating 2-10mls of urine and using the urine pellet in the MTB/RIF improves sensitivity by approximately 34%. Moreover, combining two or more urine-based diagnostic tests improves sensitivity. For instance, the combination of the urine LAM ELISA with urine MTB/RIF was able to detect approximately three quarters of all sputum scarce and smear-negative TB cases and was better than using urine MTB/RIF or the urine LAM strip test alone. In patients with CD4≤100 cells/ml the combined urine MTB/RIF and urine LAM strip test detected 87% of TB cases were sputum smear alone would detect 57%. Of the all sputum smear negative TB cases approximately half were correctly identified by the same combination.

Over the past decade there has been an increased burden of smear-negative and sputum scarce TB in HIV co-infection and high mortality rate [6]. Moreover culture takes 4-6 weeks with low sensitivity in paucibacillary cases [43]; whilst sputum smear microscopy has suboptimal sensitivity with 50-70% of HIV coinfected TB suspects being smear negative [27, 45]. This has to

misdiagnosis and delayed diagnosis of TB leading to high rates of morbidity and mortality [45]. Nevertheless, the use of MTB/RIF on urine could provide both a rapid diagnosis and rifampicin susceptibility test result for new hospital admissions within 24 hours. The advantage of urine MTB/RIF over the other urine-based diagnostics is its ability to detect rifampicin resistance.

However, there were some limitations to this study. There was no sputum MTB/RIF performed which could have been used to compare the performance of MTB/RIF using urine and sputum as samples. More so, the results of this study were not used for treatment decisions therefore it became difficult to comment on the impact of the study. In addition some of the urine samples were inadequate for centrifuging for the MTB/RIF assay. Lastly, all the urine-based diagnostic tests were performed on frozen samples and this could have had a negative effect on the test results.

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Combining the tests yielded an increased sensitivity than each test alone. There was a significantly remarkable increase in the sensitivity of combined tests within patients who were severely immunocompromised compared to patients with CD4>200 cells/ml. MTB/RIF still detected TB in the patient with PTB only; GUTB might be the highly suspected infection resulting in urine Xpert positivity in these patients due to its silent and often asymptomatic characteristics in its latent phase [44]. In the EPTB patients LAM ELISA test performed better than other tests offering even a higher sensitivity when used together with MTB/RIF. The introduction of these urine-based adjunctive tests in resource-constrained settings as a way of complementing the underperforming smear microscopy is imperative especially with urine MTB/RIF having the advantage of detecting rifampicin resistance. Centrifuging and pelleting urine has been demonstrated to improve performance in urine Xpert MTB/RIF and this conforms to previous studies that have centrifuged non-sputum samples like CSF and pleural fluid [43, 46] . It is most likely that if the urine samples in the SS group is

centrifuged and pelleted at higher gravitational force for more at least 20 minutes, the sensitivity of urine Xpert MTB/RIF will enormously increase.

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CHAPTER 5: GENERAL DISCUSSION AND RECOMMENDATIONS

5.1 Discussion

There are almost 9 million cases of TB and approximately 2 million TB-related deaths annually . South Africa has the fifth highest global TB incidence and 60% of these new cases are HIV-coinfected [16]. The biggest weakness in the management of persons with TB and HIV co-infection is the problem of mis- and delayed diagnosis [17]. The inability of these patients to produce sputum samples, the often paucibacillary disease and consequent poor performance of sputum smear-microscopy, and high incidence of extrapulmonary TB (EPTB) underpin this diagnostic problem [13]. Thus, non-sputum-based rapid and effective diagnostic tools are urgently needed TB HIV co-infection [45]. Urine, being easy to collect and readily available is an ideal target biological sample [69]. The urine LAM strip test, being a simple, rapid bedside tool, is a potentially ideal tool for use in low-resource high burden primary care settings [53]. The Xpert MTB/RIF is an exciting novel sputum-based diagnostic that may have possible utility when applied to urine. This study investigated the utility of these novel urine diagnostic tools for TB diagnosis in a high HIV prevalent setting. This study sought to clarify whether the use of a LAM strip grade-2 cut-point instead of the manufacturer's recommended cut-point improved inter-rater agreement and LAM strip rule-in performance in ambulatory patients presenting at a health-care facility. Furthermore, the study also sought to investigate if, in patients co-infected with HIV and advanced immunosuppression, the combination of urinary LAM and Xpert MTB/RIF could improve diagnosis in this vulnerable patient group.

This study shows that the use of the grade-2 cut-point and not the manufacturer's suggested grade-1 cut-point for the urine LAM strip test improved the inter-reader agreement and test specificity in an ambulatory primary care patient cohort. Moreover, since most grade-1 results obtained

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using the manufacturer's suggested grade-1 cut-point are highly likely to be associated with false positive results due to NTMs and some fungal species such as Candida; making use of the grade-2 cut-off point helps in the elimination of most of these false positives. This however, resulted in the loss of true positive grade-1 results, which is a possible reason why the sensitivity dropped down significantly [5, 65, 67]. Specificity improved significantly, but only at the expense of sensitivity. These findings are similar to those of Peter *et al.* whose study investigated urine LAM strip performance in a hospitalised HIV-infected patient cohort with advanced immunosuppression also in Cape Town, South Africa [6], but differ from those of Lawn *et al.* in an ARV-screening clinic [19]. The reason for these difference remain unclear as the HIV-infected patients evaluated in this study and that of Lawn *et al.* were similar with respect to the levels of immunosuppression. One possible explanation is that the 24 LAM strip-positive test results in the Lawn et al. study had high corresponding LAM ELISA optical densities and were thus likely to have had strongly positive LAM strip tests (grade 2-5) [88], thereby avoiding the presence of the confusing faintly positive (grade-1) bands. Unfortunately though, given the very low sensitivity of the urine LAM strip test (24% in HIV-infected) in this out-patient cohort, even cut-point optimization to improve rule-in test utility could only marginally improve PPV. The low sensitivity of the urine LAM strip test in this patient cohort was consistent with a number of studies performed with the LAM ELISA-kit in similar patient populations [65, 73, 87, 89]. Thus, it seems unlikely that the urine LAM strip will offer significant clinical utility in ambulatory primary care clinic patients with suspected TB, even if they are HIV co-infected.

Despite the limited performance of urine LAM in ambulatory patients which this study confirms using the LAM strip test, studies of urine LAM (both the ELISA and now recently the strip test) show moderate sensitivity (~50%) amongst severely-ill hospitalised TB HIV co-infected patients with advanced immunosuppression. Higher urine LAM concentrations have been found to be correlated with disseminated TB and higher total bacillary burdens [71].

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HIV co-infected patients with advanced immunosuppression have high TB-related mortality and often present with disseminated disease which is sputum scarce or sputum smear-negative [90]. They are a particularly vulnerable, diagnostically challenging patient sub-group in whom prompt diagnosis and the early initiation of TB treatment may save lives [90]. Novel non-sputum diagnostic approaches are urgently needed and consequently, we hypothesized that the use of urine-based Xpert MTB/RIF alone or in combination with urine LAM strip testing may offer important clinical utility in hospitalized patients with suspected TB HIV co-infection and sputum scarce or smear-negative disease [45].

This study found that: i) urine-based MTB/RIF offered equivalent sensitivity to urine LAM (ELISA or strip test) in TB HIV co-infected patients with sputum scarce or smear-negative disease with the additional advantage of rifampicin drug susceptibility testing; ii) a combination of urine LAM ELISA and MTB/RIF testing could detect close to three quarters of sputum scarce *M. tuberculosis* culture positive patients; and iii) the pelleting of 2-10ml of urine prior to MTB/RIF testing significantly improves sensitivity [45]. Sputum-based Xpert MTB/RIF is currently being implemented for frontline TB diagnosis in South Africa as a replacement for smear microscopy [32, 34, 45, 82], however, few data on the Xpert MTB/RIF performance in urine and other non-sputum samples is available. This study indicates that urine MTB/RIF could be considered as an alternative diagnostic tool in HIV-infected hospitalised patients with suspected TB who are unable to produce sputum or have EPTB. Used in combination with urinary LAM, a diagnosis could be provided for close to three quarters of diagnostically destitute patients within 24 hours of admission.

Although urinary *M. tuberculosis* culture is of little use amongst ambulatory HIV un-infected patients, a few studies in HIV-infected patients with advanced immunosuppression have found the diagnostic yield of repeated urinary culture to be approximately 75% [4], although the sensitivity of

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urine-based smear-microscopy, even in HIV-infected patients is dismal. In this study we found the sensitivity of urine-based Xpert MTB/RIF to be 48%, and significantly improved by the centrifugation of up to 10mls of urine. This result is not unsurprising given the known comparative limits of detection between urine culture (~10000 organisms/ml), sputum culture (10-100 organisms/ml), Xpert MTB/RIF (~131 organisms/ml) and smear-microscopy (10000 organisms/ml) [34, 81, 88, 91, 92].

Similar studies on the performance of urine LAM and Xpert MTB/RIF were also carried out by Wood *et al* (2012). The study showed that approximately 50% of all urine LAM ELISA positive patients were also urine Xpert MTB/RIF positive [34, 93]. The studies further suggested that since Xpert MTB/RIF only detects whole *M. tuberculosis* bacilli, the Xpert MTB/RIF positive results together with LAM antigenuria indicates strong possibilities of genitourinary TB [34, 93]. This study confirms the possibility of LAM antigenuria resulting directly from genitourinary TB where the LAM antigen passes directly into the urine without crossing the renal glomerular basement membrane [45, 93]

It has been shown that the TB LAM Ag tests offer particular POC rule-in utility in hospitalised HIV infected patients with advanced immunosuppression [6], and have been proposed as a rapid tool to guide the rapid initiation of anti-TB therapy. In this study we demonstrate the potential use of urine Xpert MTB/RIF as an alternative rapid rule-in test for these hospitalised patients with advanced HIV-infection. Furthermore, the improved rule-in utility of the combination of urine Xpert MTB/RIF and TB LAM Ag is demonstrated. By contrast, the performance characteristics of the TB LAM Ag in our ambulatory patient group demonstrate that the urine LAM strip tests perform poorly as either a rule-in or rule-out test in this setting.

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5.2. Study limitations

Notwithstanding the exciting findings from this study, there were some limitations that need to be noted. The first arm of the study, that analysed the inter-reader agreement and performance of the urine LAM strip using two different cut-points in the out-patient department (OPD) patients, used data that came from a single study site. Moreover there were fewer HIV positive patients (n=74) compared to HIV negative patients (n=200). Intra-reader agreement was not done and evaluated. On the other hand, the second arm of the study had a shortfall of inadequate sample volumes. Hence many other procedures, such as pelleting urine samples that could have given better diagnostic performance were not carried out. Only 38/59 Xpert MTB/RIF negative samples were centrifuged and resuspended for the urine Xpert MTB/RIF, of which the volume used for the pelleting of samples ranged between 2-6ml due to the inadequate volumes. Some urine samples were missing. Only the sensitivity of the test was used as the diagnostic performance parameter. Urine Xpert MTB/RIF's specificity would have been useful in the decision on whether to use the test as a 'rule in' or not. Furthermore, there was no sputum Xpert MTB/RIF done and this could have helpful in the comparison of urine based Xpert MTB/RIF and sputum based Xpert MTB/RIF.

5.3. Recommendations

There is a need of carrying out further studies to clarify and substantiate the findings of this study. A larger study cohort will be more representative of the situation on the ground rather than a smaller sample size that was used. The first arm of the study was done in a single community; it is therefore recommended that more study sites be established in different parts of the Sub-Saharan Africa in order to get a clearer picture of the real situation. There is a great need of optimisation of the urine Xpert MTB/RIF before carrying out any investigation of study patients. This could involve the use of urine from healthy subjects; these urine samples could be spiked with

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different concentrations or colony forming units (CFUs) of *M. tuberculosis*. The spiked urine samples could be used to find out the limit of detection (LOD) of urine Xpert MTB/RIF. The same samples could be used to optimise the correct urine volume and speed or gravity of centrifugation. There is also need of using fresh urine samples rather than urine samples that have been archived in the fridge for long periods of time. Large volumes of urine samples would be necessary to carry out the further investigations.

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