



**Early morning urine collection to improve the sensitivity of LAM in
hospitalised TB/HIV co-infected patients**

By

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RESEARCH OUTPUT

¹Phindile Gina, ¹Philippa J. Randall, ¹Tapuwa Muchinga, ¹Keertan Dheda¹ and Jonny G. Peter (2015). *Early morning urine collection to improve the sensitivity of LAM in hospitalised TB HIV co-infected patients*. PLoS ONE, submitted.

DECLARATION

I, NTOMBENHLE PHINDILE GINA, 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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DEDICATION

This thesis is dedicated to my parents Mr. Jeffrey and Mrs. Nomsa Gina who always stood by me and offered invaluable motivation, support and encouragement throughout the entire research study.

ACKNOWLEDGEMENTS

First and foremost I would like to express my sincere gratitude to my supervisor and mentor, Dr Jonathan G Peter (Lung Infection and Immunity Unit), for his invaluable supervision, expertise, assistance and guidance in developing this research project. It is also with immense gratitude that I acknowledge the support

and help of my co-supervisors, Professor Keertan Dheda, Dr Philippa Randall and Mr Tapuwa Muchinga, whose expert knowledge and assistance has helped make this study a success.

ABSTRACT

Background

Point-of-care detection of urine lipoarabinomannan (LAM) is a low-cost rapid TB diagnostic for use in HIV co-infected patients. However, its sensitivity in these patients is suboptimal. Strategies to improve its performance is a need. The hypothesis was that early morning urine (EMU), rather than random urine sampling, would improve LAM's sensitivity.

Methods

Recruitment process conducted between June 2012 and February 2014 for HIV-infected patients from four hospitals in Cape Town, South Africa presenting with possible TB (all patients initiated on TB treatment). Fresh random and early morning urine (EMU) samples (~10-30 ml) collected in sterile containers. Following the manufacturer's instructions, an Alere Determine® TB Lateral flow assay performed on each sample, using both grade 1 and 2 cut-points. A single sputum Xpert MTB/RIF and/or liquid TB culture was a reference standard. Those designated probable TB patients were sputum Xpert MTB/RIF and/ TB culture negative, but started on TB treatment.

Results

Of 184 HIV- infected adults screened, only 123 patients commenced anti-TB treatment and provided matched random and EMU samples. 33% (41/123) and 67% (82/123) had definite TB and probable TB, respectively. Amongst definite TB, LF-LAM sensitivity, using the grade 2 cut-point, increased to 39% (95% CI: 26-54, 16/41) from 12% (95% CI: 5-24, 5/41) using EMU versus random, respectively (p=0.004). Similarly, amongst probable TB, urine LAM sensitivity increased to 24% (95% CI: 16-34, 20/82) (p=0.01) from 10% (95% CI: 5-17, 8/82). Age, CD4 cell count, Xpert MTB/RIF/liquid TB culture positivity and prior initiation of anti-TB treatment within 7 days were not significant predictors of an increasing urine LAM grade between random and EMU specimens. LF-LAM specificity was not determined.

Conclusions

EMU sampling can improve the sensitivity of LF-LAM testing for hospitalised TB/HIV co-infected patients. There is a need for further research is to assess if this additional diagnostic sensitivity can affect patient-important outcomes.

ABBREVIATIONS

Ag	Antigen
AIDS	Acquired Immunodeficiency Syndrome
CI	Confidence Interval
ELISA	Enzyme-Linked Immunosorbent Assay
EPTB	Extrapulmonary tuberculosis
HIV	Human Immunodeficiency Virus
LAM	Lipoarabinomannan
LAM-ELISA	Lipoarabinomannan -Enzyme Linked immunosorbent assay
LF	Lateral flow
LF-LAM	Lateral Flow - Lipoarabinomannan
MDR TB	Multi-Drug Resistant tuberculosis
<i>M.tb</i>	<i>Mycobacterium tuberculosis</i>
NAAT	Nucleic Acid Amplification Test
PCR	Polymerase Chain Reaction
POC	Point-of-care
PTB	Pulmonary tuberculosis
RIF	Rifampicin
TB	Tuberculosis
USA	United States of America
WHO	World Health Organisation
XDR TB	Extremely Drug Resistant tuberculosis

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CHAPTER 1: LITERATURE REVIEW

Introduction

Tuberculosis (TB) is an enormous global health problem. Annually approximately 9 million people develop TB, and over 1.7 million deaths are attributed to TB (1). South Africa is one of the countries with the highest burden of TB. Worldwide, South Africa has a higher incidence than India and China, and this has increased by 400% over the past 15 years (2).

World Health Organization (WHO) statistics estimate South Africa to have an incidence of 450,000 cases of active TB per annum (1), representing approximately 1% of its population. Of the 450,000 new cases WHO estimates that approximately 270,000 (60%) are co-infected with the human immunodeficiency virus (HIV). The latest figure from the South African Department of Health is that 73% of adult TB patients are HIV positive (2). When a patient is infected with both *Mycobacterium tuberculosis* (*M.tb*) and HIV, the two pathogens interact synergistically (Figure 1) speeding the progression of illness and increasing the likelihood of death (3). The presence of HIV makes a person more vulnerable to developing TB, and having TB accelerates the progression of HIV infection (3). TB is the most common opportunistic infection among persons presenting with HIV. Furthermore HIV-infected patients with TB are at a higher risk of death (3, 4).

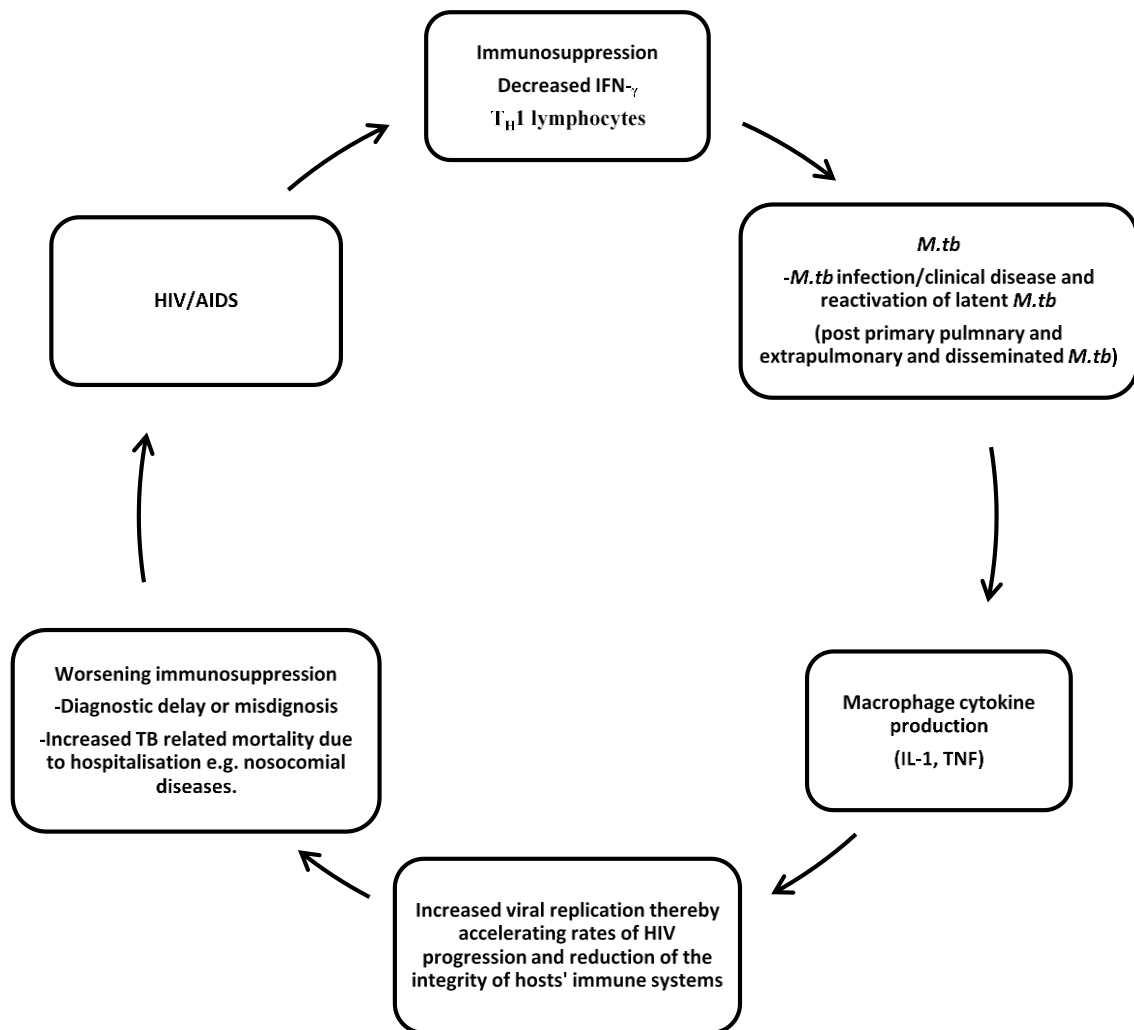


Figure 1. The synergy of HIV and TB

The low case-detection rate is largely due to reduced capacity amongst resource-limited national TB programmes. It is compounded by ineffective diagnostic algorithms that incorporate decade-old tests with sub-optimal diagnostic accuracy (5). A delay in TB diagnosis is associated with high mortality even in richly-resourced settings (6).

1.1. Diagnosis in HIV/ TB co-infection

In the majority of disease-endemic countries, sputum microscopy, a method introduced over a century ago, is still the first line diagnostic test for TB. In adult pulmonary TB without immune suppression it has reasonable sensitivity compared to culture (7). However, for detection of TB in children, TB/HIV co-infected patients and extra-pulmonary TB (EPTB)

its sensitivity drops to unacceptable levels (8). In cases of TB in children, smear microscopy needs to be performed on paucibacillary non-sputum based biological fluids (8).

In HIV co-infection, reduced smear microscopy sensitivity is the result of a failed inflammatory response with consequent reduced caseating necrosis and lower numbers of acid-fast bacilli in the airway (9). Another diagnostic problem in severely ill TB/HIV co-infected patients is that they are often unable to self-expectorate sputum or have extra-pulmonary disease. Acquiring a specimen for TB diagnosis in these patients therefore requires assisted sputum sampling e.g. via hypertonic saline induction or alternative invasive sampling (10). In many TB/HIV endemic countries, capacity for assisted and invasive sampling is limited or unavailable. Furthermore, laboratory capacity for culture-based diagnosis is limited. Thus, especially in settings with dual epidemics of HIV and TB, the lack of accurate and rapid diagnostic TB testing is a major impediment to achieving global TB control. There is a great need to develop alternative rapid-diagnostic methods that are more sensitive and specific than smear microscopy and better able to detect TB disease at different sites the body (6).

Global TB diagnostic pipeline			
	Early Development	Late or Completed Development	On Pathway to WHO Evaluation
HIGH COMPLEXITY ASSAYS	Molecular Detection/DST		
	TruArray MDR-TB (Akkoni) COBAS TaqMan MTB +DST(Roche) Hydra 1K (insilixa) Mycobacterium Real-time MDR (CapitalBio)	TRC Rapid MTB (Tosoh) VereMTB (Veredus Laboratories) LiPA Pyrazinamide (Nipro) LATE-PCR Lights on / Lights off (Hain) TBMDx (Abbott) Meltpro (Zeesan) Mycobacteria RT PCR (CapitalBio) REBA MTB-XDR (YD Diagnostics) EasyNAT TB (Ustar) BD Max (BD)	GenoTYPE MTBDRsl (Hain) LiPA MDR-TB (Nipro) REBA MTB-Rifa (YD Diagnostics)
	Culture-based Technology		
	BNP Middlebrook (NanoLogix) Rapid colorimetric DST	TREK Sensitive MYCOTB (Trek)	
MODERATE COMPLEXITY ASSAYS	Molecular Detection/DST		
	Xpert Ultra and Xtend XDR (Cepheid) Alere Q (Alere) Enigma ML (Enigma Diagnostics) Q-POC (QuantuMDx) EOSCAPE (Wave80) RT-PCR Testing Platform (NWGHF/Guidel) iCubate 2.0 (iCubate) TBDx system (KGI) DiagCORE (STAT Diagnostica) LabChip G2-3 (Nanobiosys)	Genedrive MTB/RIF (Epistem) Truelab/Truenat MTB (Molbio)	TB LAMP (Eiken)
	Volatile Organic Compounds		
	BreathLink (Menssana) Prototype breathalyzer (Next Dimensions) TB Breathalyser (Rapid Biosensor Systems) Aeonose (The eNose Company) Breath analysis instrument (Metabolomx)	Giant African Pouch Rats (Apopo)	
Automated Microscopy & Imaging			
TBDx (Applied Visual Sciences) Fluorescent microscopy (ID-FISH Tech.) Automatic TB Screener (Fluorobot)	Microimager (BD) CAD4TB (Delft Imaging Systems)		
LOW COMPLEXITY ASSAYS	Antigen & Antibody Detection		
	LAM in sputum (Standard Diagnostics) Multiplex antibody array (mBio)		Alere Determine TB-LAM in urine (Alere)
	Enzymatic Detection		
	β -lactamase reporter (Global BioDiagnostics)		

Source: FIND, Geneva

Figure 2: The global TB diagnostics pipeline in 2015 (11).

The development of the Xpert MTB/RIFXpert (Cepheid, Sunnyvale, CA, USA) assay, a nucleic acid amplification test (NAAT) able to detect the presence of both *M.tb* complex DNA and rifampicin drug resistance (strongly correlated with MDR-TB) in less than 2 hours, provided a major advance in TB diagnosis (Figure 3). The WHO endorsed the use of the Xpert MTB/RIF assay for frontline TB diagnosis in HIV-infected and MDR-TB suspects in December 2010 (12). When compared to smear microscopy the Xpert MTB/RIF assay provides clear clinical benefits for the early diagnosis of suspected pulmonary TB cases (13). The Xpert MTB/RIF assay requires sputum for testing and has reduced performance in HIV-infected compared to uninfected patients and on extrapulmonary compared to sputum

samples (13). Despite international efforts to reduce costs, a single test cartridge still costs ~US\$9.98 and a four-cartridge machine ~US\$17 000, making implementation unaffordable at present in a number of high burden settings. The Xpert MTB/RIF assay cannot differentiate between the presence of viable or dead bacilli, increasing the risk of false positive results amongst patients with previous TB infection, common in HIV co-infected patients with advanced immunosuppression (14, 15). The Xpert MTB/RIF assay is not a true point-of-care test. Large studies using the Xpert MTB/RIF assay in primary care clinics, showed that the test increased the number of individuals with bacteriologically confirmed TB, and reduced time to treatment initiation particularly among HIV-infected participants (16). However, the use of the Xpert MTB/RIF assay did not affect TB-related morbidity or mortality (16). Nevertheless, the Xpert MTB/RIF assay is a major advance on smear microscopy, especially for HIV co-infected patients with advanced immunosuppression and possible TB, non-sputum based, inexpensive point-of-care diagnostics are still urgently required.

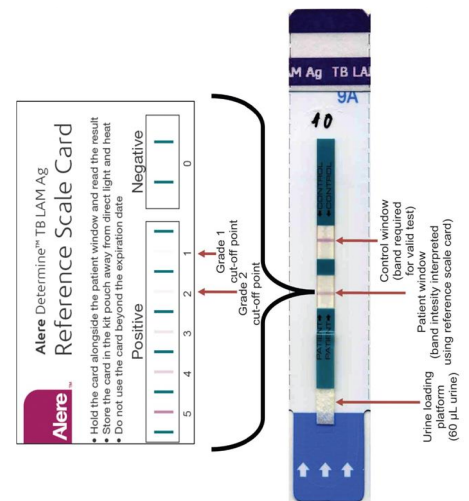
TB culture remains the gold standard diagnostic test for TB in both immunocompetent and compromised patients. Amongst HIV-infected patients it is often the only method of confirming TB. Consequently, the WHO and affiliated organisations such as FIND diagnostics continue to mobilise international resources to strengthen culture capacity in high burden resource-limited settings. Culture is also a critical diagnostic modality in settings of high MDR and XDR-TB, however there is a need for culture based technology for extended drug sensitivity testing (17). TB culture , takes up to 6 weeks to provide results, and requires considerable laboratory infrastructure (18). In the context of severe TB disease, treatment decisions are rarely based on culture results, a finding illustrated in a number of studies (16).



A. Fluorescence LED Microscopy



B. Automated integrated NAATs-Xpert MTB-RIF



C. Immunochromatographic strip test e.g. Urine LAM strip test (Determine TB LAM Ag test, Alere)

The progress of frontline TB diagnosis from smear microscopy to molecular methods and onwards toward point-of-care test formats (19)

For decades, researchers and the industry had pinned their hopes on serological antibody-detection methods for a bedside TB test. Consequently, TB immunodiagnostics, such as serological tests, tuberculin tests (TSTs) and interferon- γ release assays (IGRAs), have been extensively studied (20). The failure to develop antibody-based TB tests that meet clinical needs does not imply that biomarker approaches should be abandoned. For instance, *M.tb* metabolites can be detected as volatile organic compounds (VOCs) in breath, using gas chromatography/mass spectrometry-based analysers (21). Recently, a breath test identified biomarkers of active pulmonary TB with 85% accuracy in symptomatic high-risk subjects (22). In addition, Banday *et al* demonstrated the potential utility of urine VOCs in the diagnosis of TB disease (23). VOC detection offers potential advantages, but they remain under development and a long way from clinical use.

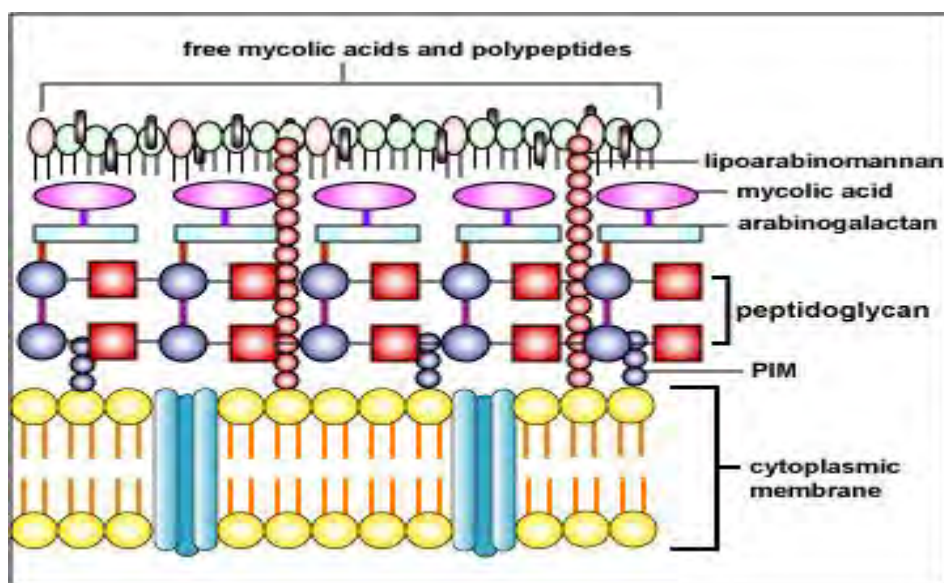
The use of urine, rather than sputum, for diagnostic testing provides an appealing alternative. Collection is easy and less invasive, it is readily available, and its collection presents a very low infection risk to staff. In fact, urine antigen detection is the most common diagnostic technique employed for a number of infectious diseases e.g. *Leginella*. Its use for TB diagnosis is a focus of research and development. Lipoarabinomannan (LAM) is the most extensively evaluated and promising, and is commercially available as a bedside test (24).

1.2. Urine LAM for TB diagnosis

1.2.1 What is Lipoarabinomannan (LAM)?

LAM is a heat-stable 17.5kD glycolipid that forms one of the main components of the outer cell wall of mycobacterial species (Figure 4). It is a heterogeneous immune-reactive glycoconjugate (25), that accounts for up to 15% of the total bacterial weight and is an important virulence factor of *M.tb* (26).

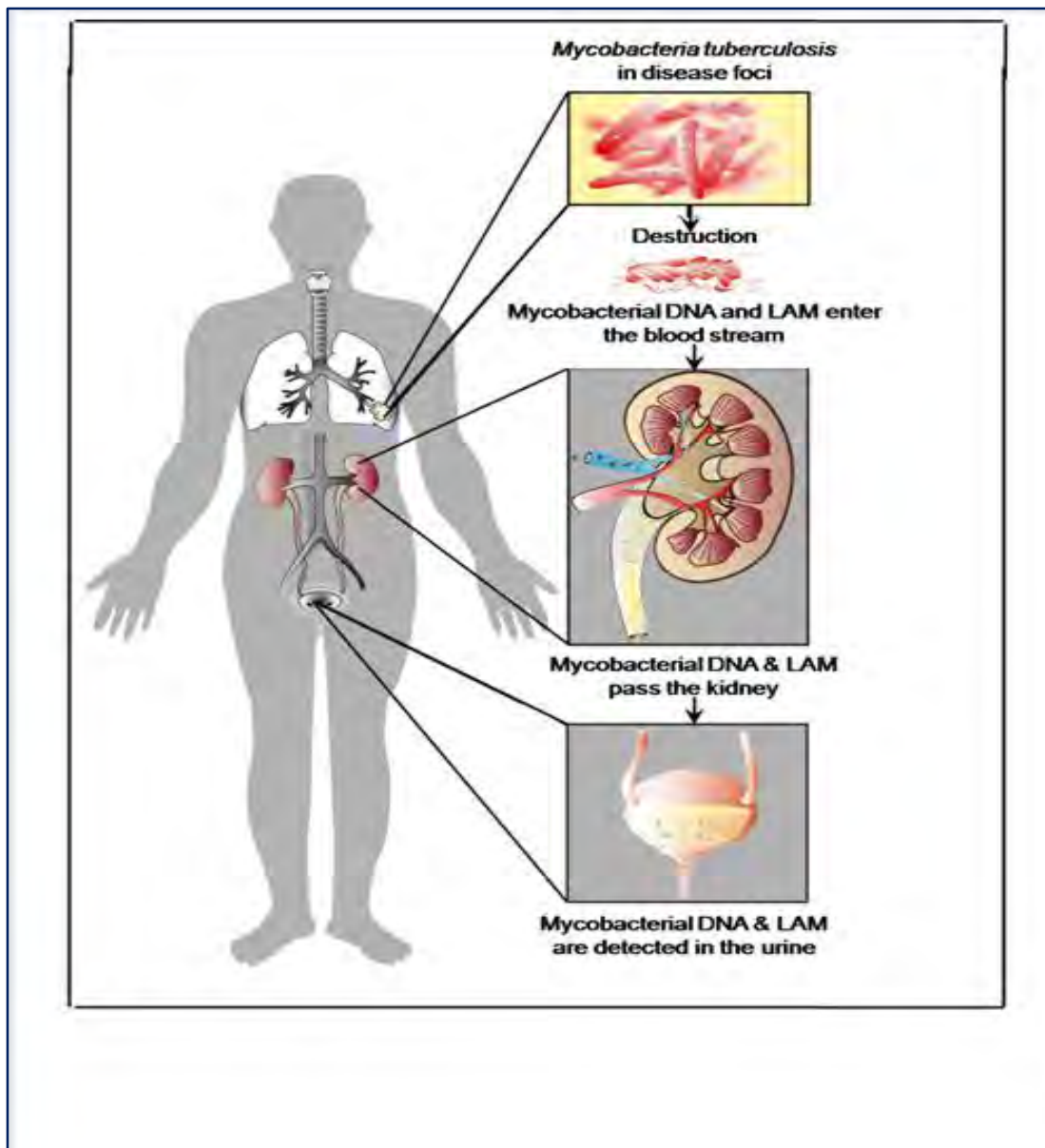
LAM consists of three distinct structural domains: a phosphatidylinositol (PI) anchor, a branched mannan and a branched arabinan (24).



Simplified illustration of the *M.tb* cell wall, showing the position of LAM (27)

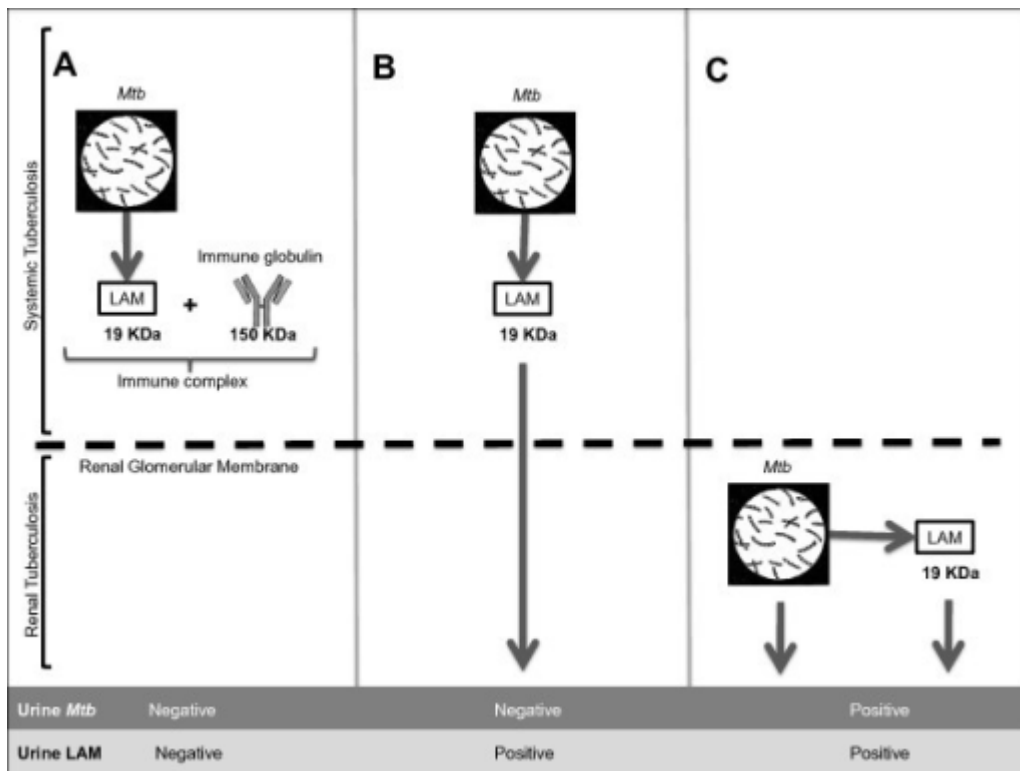
1.2.2 Why is LAM detected in the urine?

LAM can be detected in the urine of patients with active TB. It travels in the bloodstream and passes through the renal filtration barrier without major changes, thus being detectable in an antigenically intact form in urine (Figure 5) (28) .



Annotated diagram, illustrating the passage of mycobacterial DNA and LAM antigen from its infection site to expulsion in the urine (25)

LAM is detectable in highly varying concentrations in the urine of TB patients following the highly variable sensitivity of the LAM assay in different groups of TB patients. Most notably, LAM is found in the highest concentrations amongst HIV infected patients with advanced immunosuppression and disseminated TB.



Three proposed models showing the possible fate of LAM molecules released from systemic or urinary *M.tb* organisms (29)

The current thinking about the mechanisms by which LAM concentration is affected in urine are depicted in Figure 6. These include:

- A. Systemically-released LAM binds to an antibody forming an immune complex that is not able to pass across the glomerular membrane, and is therefore not present in urine. This model may account for the failure to detect LAM in the urine despite the presence of pulmonary *M.tb*. or a low bacillary burden.
- B. Circulating LAM, unattached to a specific anti-LAM antibody, is freely filtered through the kidney into the urine, giving rise to a positive LAM test if antigen concentration saturates mechanism A (high bacillary load).
- C. *M.tb* within the renal tract releases LAM directly into the urine, which gives rise to a positive LAM test in the presence of *M.tb* organisms in the kidney (29).

This model explains, in part, why LAM has been found in higher concentration in the urine of patients with advanced HIV infection and disseminated forms of TB as a high bacillary load would lead to a higher antigenemia (30). Additionally, increased LAM detection has

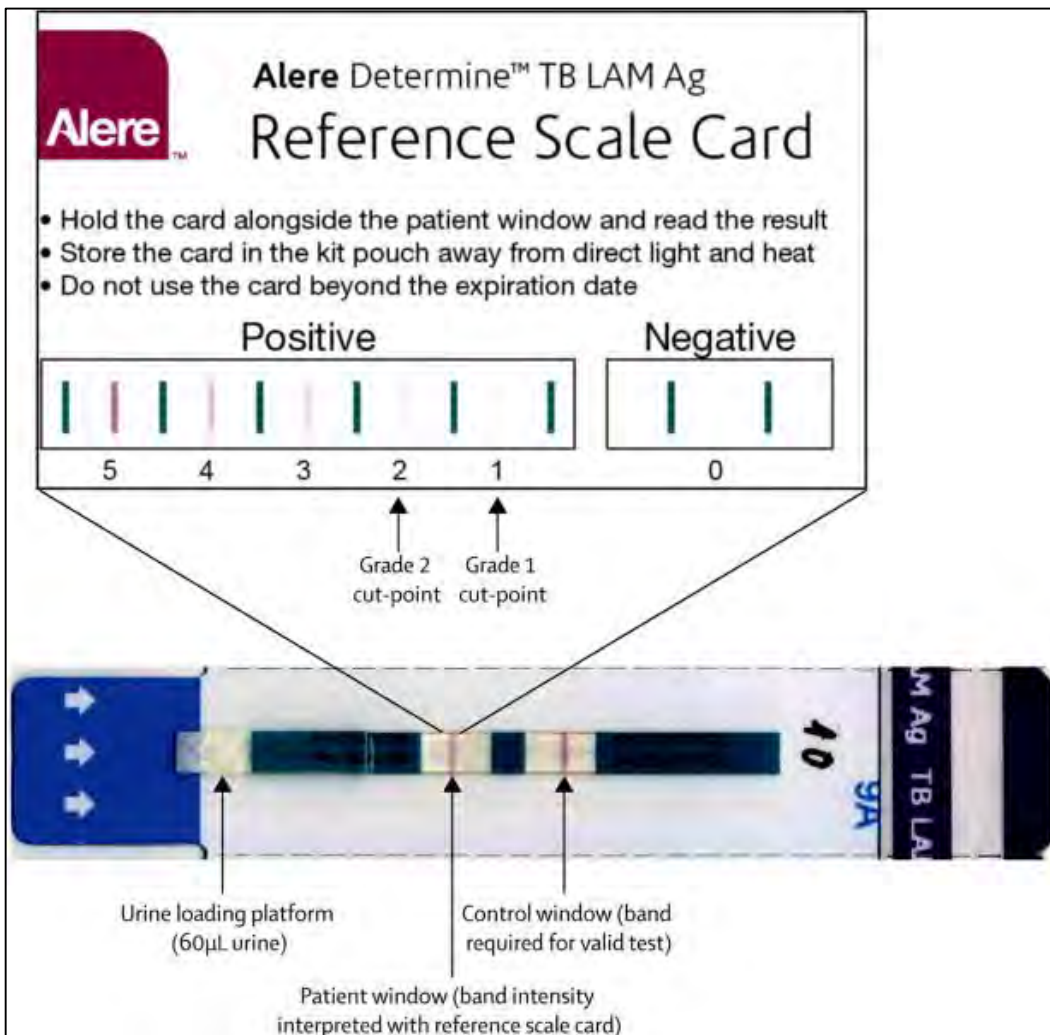
been noted in patients with abnormal renal function due to the presence of TB bacilli in renal parenchyma (29). This finding was supported by the detection of bacilli in the urine by Xpert MTB/RIF (31). Alterations in the podocyte barrier (filtration barrier), with a resultant increase in glomerular permeability could also increase urinary LAM concentrations.

1.2.3 Development of the LAM-ELISA

In 2001, Hamasur and colleagues developed an ELISA method for the detection of LAM, using a polyclonal anti-LAM antibody, and found LAM to be detectable in mouse urine, post *M.tb* intraperitoneal injection (32). The methods were highly sensitive, detecting LAM at concentrations of 1 ng/ml and 5 pg/ml (32). In 2005, the pre-commercial prototype of the LAM ELISA (*M. tuberculosis* LAM ELISA Test[®], Chemogen, and Portland, USA) was first tested, using human urine specimens in a Tanzanian cohort of TB patients (33). This pre-commercial version was superseded by the development of a second generation, commercially-available prototype known as the Clearview[®] TB ELISA (Inverness Medical Innovations, USA) and retailed as the TB LAM ELISA (Alere, USA; henceforth called LAM-ELISA), using the original polyclonal anti-LAM antibody.

1.2.4 Development and use of the LAM lateral flow assay

In 2008, Alere Medical Innovations developed a simple lateral flow format, using the same polyclonal anti-LAM antibody coupled to gold nanoparticles (34). The Alere Determine[™] TB LAM Antigen lateral flow assay (Alere, USA; henceforth called the LF-LAM test) was the first commercially available true point-of-care TB test, requiring only 60 µl of unprocessed urine (Figure 7).



Alere Determine™ TB LAM Ag test. 60 µls of urine is applied to the urine loading platform (left arrow). After a 25 minute incubation period, the colour intensity of the patient window band (middle arrow) is graded (0 – 5) using the test’s reference scale card, so determining a patient’s TB status (35).

The test costs less than US\$3 can be performed at the patient’s bedside, thus obviating the use of laboratory infrastructure and trained personnel. The test, shown in Figure 7, is performed by applying 60 µls of urine to the loading platform, incubating it at room temperature for 25 minutes (36), then comparing the colour intensity of the band in the patient window against those on the ‘reference scale card’ supplied by the manufacturer. Colour intensity ranges from 0 - 5. The test is only valid if a band appears in the control window.

Initially, the manufacturer categorised patients with grade 0 as test ‘negative’, whereas grades 1 to 5 were classified as test ‘positive’. However, in a test where rule-in utility needs to be optimised due to sub-optimal sensitivity, the grade-1 cut-point was found to lack specificity

and have decreased inter-observer agreement (37). Consequently, in early 2014 the manufacturer removed the old grade 1 colour category replacing it with the original grade 2 colour category, and adjusting the other categories accordingly, so reducing the range to five (0 - 4).

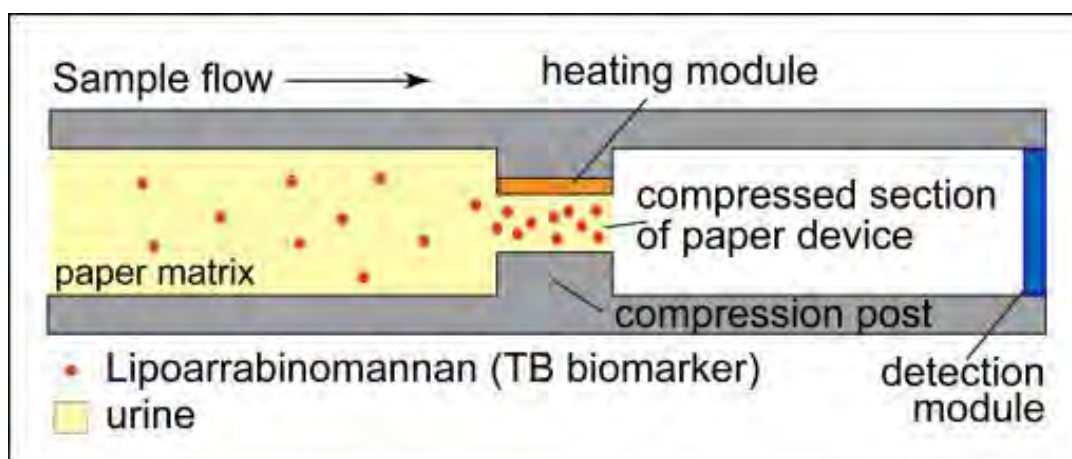
1.2.5 LAM ELISA diagnostic accuracy

The LAM-ELISA for TB has been extensively evaluated (Table 1). Apart from the early studies by Boehme *et al.* in 2005 (33) and Tessema *et al.* in 2002 (38), the overall sensitivity of LAM-ELISA has been modest, ranging from 13% - 51% for the diagnosis of active pulmonary TB (26). However, amongst HIV-infected patients with advancing immunosuppression (lower CD4 cell counts) and hospitalised in-patients with higher burdens of disease, urine LAM-ELISA sensitivities improved to potentially useful levels (31). In addition, incremental sensitivity over sputum smear microscopy was noted, such that the combination of sputum smear microscopy and LAM-ELISA was able to detect as many as 75% of TB in patients with $CD4 \leq 200$ cells/mm³ (37). Thus, urine LAM-ELISA seemed to offer the greatest potential as an add-on TB diagnostic for HIV co-infected patients with advanced immunosuppression (39). This offers particular utility given that sputum-based conventional and novel diagnostic tools perform poorly in these vulnerable patients where EPTB and disseminated TB is common and TB-related mortality as high as 50% (40). The development of the LAM-ELISA into an inexpensive lateral flow assay format (LF-LAM) further improved its potential utility as it enabled rapid results at a patient's bedside.

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

Table 1. Performance of standardised urine LAM ELISA assays in different clinical subgroups with suspected TB stratified by HIV status and, where available, CD4 T cell count (25).

Hypothesis is that heat and mechanical compression of the paper matrix containing the urine sample - herein referred to as compressive evaporation - would cause urine to evaporate and LAM to accumulate, thus concentrating LAM at the heat source (Figure 8) (38). An additional method under investigation is heating. The drawback of all these methods is the requirement for laboratory infrastructure and resources, all costs that nullify the bedside nature of the LF-LAM test format.



Schematic illustration of the compressive evaporation technology for sample application, evaporation and LAM concentration (49).

A simple, useful strategy proposal: use the natural urine concentration method that occurs overnight, by collecting urine samples in the morning. Early morning urine (EMU) collection (after an individual awakes from sleep) is generally accepted as a more reliable method for detecting most antigens, since it corresponds closely with 24-h urine collection (50). A 24-h urine collection is considered the gold standard in studies evaluating urinary analytes, such as protein creatinine ratio for kidney disease. However, some studies that compared measurements of a EMU to a 24-h urine collection to measure albumin creatinine ratio (ACR), showed EMU to possess a higher sensitivity possibly due to the difficulties of conducting accurate 24 hour collections (51). Twenty-four hour urine collection is cumbersome. A practical and easier alternative is the collection of an EMU sample. It is a suggestion that EMU is preferred over a random urine sample (daytime random sample) because the former is less influenced by factors such as hydration status and physical activity that can reduce variability of urine analyte concentration (50, 52).

As with analytes, so with antigens or microorganisms. Prolonging the period between voiding improves total antigen excretion, so improving detection. EMU samples have also been a means of obtaining a microbiological diagnosis of TB. Likewise, urine-based PCR for *M.tb* has shown promise in TB/HIV co-infected patients (53). However data on the sensitivity of microbiological TB diagnosis using EMU is limited and available data suggests limited smear and culture positivity (54).

The proposed hypothesis is that EMU sampling would increase urine LAM sensitivity compared to random sample collection. In addition, considering the treat-to-test hypothesis (55), it was considered important to explore whether initiation of empiric anti-TB treatment prior to EMU LAM would further improve sensitivity.

CHAPTER 2: Journal Ready ManuscriptEarly morning urine collection to improve the sensitivity of LAM in hospitalised TB HIV co-infected patients

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ABBREVIATED TITLE

Early morning urine and LAM test-strip sensitivity

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TB HIV co-infection, TB-Determine LAM lateral flow strip, early morning urine (EMU)

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ABSTRACT

Background

Point-of-care detection of urine lipoarabinomannan (LAM) is a low-cost rapid TB diagnostic for use in HIV co-infected patients. However, its sensitivity in these patients is suboptimal. Strategies to improve its performance is a need. The hypothesis was that early morning urine (EMU), rather than random urine sampling, would improve LAM's sensitivity.

Methods

Recruitment process conducted between June 2012 and February 2014 for HIV-infected patients from four hospitals in Cape Town, South Africa presenting with possible TB (all patients initiated on TB treatment). Fresh random and early morning urine (EMU) samples (~10-30 ml) collected in sterile containers. Following the manufacturer's instructions, an Alere Determine® TB Lateral flow assay performed on each sample, using both grade 1 and 2 cut-points. A single sputum Xpert MTB/RIF and/or liquid TB culture was a reference standard. Those designated probable TB patients were sputum Xpert MTB/RIF and/ TB culture negative, but started on TB treatment.

Results

Of 184 HIV- infected adults screened, only 123 patients commenced anti-TB treatment and provided matched random and EMU samples. 33% (41/123) and 67% (82/123) had definite TB and probable TB, respectively. Amongst definite TB, LF-LAM sensitivity, using the grade 2 cut-point, increased to 39% (95% CI: 26-54, 16/41) from 12% (95% CI: 5-24, 5/41) using EMU versus random, respectively ($p=0.004$). Similarly, amongst probable TB, urine LAM sensitivity increased to 24% (95% CI: 16-34, 20/82) ($p=0.01$) from 10% (95% CI: 5-17, 8/82). Age, CD4 cell count, Xpert MTB/RIF/liquid TB culture positivity and prior initiation of anti-TB treatment within 7 days were not significant predictors of an increasing urine LAM grade between random and EMU specimens. LF-LAM specificity was not determined.

Conclusions

EMU sampling can improve the sensitivity of LF-LAM testing for hospitalised TB/HIV co-infected patients. There is a need for further research is to assess if this additional diagnostic sensitivity can affect patient-important outcomes.

Introduction

The HIV pandemic has fueled a resurgence of tuberculosis (TB), the leading cause of death in HIV-infected persons in sub-Saharan Africa (1). Rapid initiation of TB treatment may reduce mortality in these vulnerable patients, making early diagnosis an imperative (36, 56). However, there are several hurdles to diagnosis including lack of sputum production, sputum bacillary concentrations below the detection threshold of same day diagnostic tests, and atypical clinical presentation. High frequency of extra-pulmonary (EPTB) and disseminated forms of TB with advancing immunosuppression is aggravated.

Thus, there is a urgent need for new, accurate, and rapid non-sputum-based TB diagnostics that have high sensitivity in patients with HIV/TB co-infection with advanced immunosuppression (57). The Alere Determine®-TB LAM Ag lateral flow assay (Alere, USA; referred to as LF-LAM) that detects urine lipoarabinomannan, a *M.tb* cell wall-associated glycolipid, is the most promising, and only commercially available option (52). LAM is a point-of-care test that is simple to use, requires no instruments, provides a result in just 25 minutes and is low cost (<US \$3.50 per test). Importantly, it is non-sputum based and is therefore useful for patients who cannot produce sputum (29). A LAM-guided treatment strategy has recently been shown to reduce mortality in hospitalized HIV-infected inpatients with suspected TB (58). However, LF-LAM sensitivity remains sub-optimal (40- 60% in HIV co-infected patients with a CD4 count < 100 cells/ml) (59). Thus, strategies to improve sensitivity are urgently required.

Early morning urine (EMU) is accepted as a useful method for improving the concentrations of a number of antigens and analytes found in human urine (57, 60). The strategy has been shown to improve the yield of urine culture for TB diagnosis (61). Thus, we hypothesised that collecting early morning urine compared to a random spot urine specimen may be a simple and effective strategy to improve the sensitivity of LAM in hospitalised HIV-infected patients with possible TB.

STUDY POPULATION AND METHODS

The study population comprised HIV-infected adults (>18 yrs.) prospectively recruited between June 2012 and February 2014 from four hospitals: New Somerset, Victoria, Mitchells Plain and Groote Schuur Hospital, in Cape Town, South Africa. HIV-infected patients were referred for screening by emergency room or hospital doctors, if they were HIV-infected, older than 18 years and suspected to have TB. They were enrolled if they: 1. Provided informed consent, 2. Were able to provide both random and early morning urine sample, and 3. Initiated anti-TB treatment either based on clinical and radiological findings, or sputum-based smear or Xpert MTB/RIF positivity. The exclusion criteria was as follows: 1. all rapid sputum-based TB diagnostics were negative and no treatment was initiated based on clinical and radiological findings, and 2. they were unable to provide matched random and early morning urine samples.

All patients enrolled provided written informed consent and had basic clinical information collected, including demographics, past history of TB, presenting symptoms and vital signs. The University of Cape Town Human Research Ethics Committee (HREC REF 720/2013) approved the study

Tuberculosis case definitions

The reference standard for TB was single sputum sample positive liquid TB culture and/or Xpert MTB/RIF assay. Definite TB required a clinical presentation compatible with TB, initiation of anti-TB treatment by the attending clinician, with any sample *M.tb* culture or Xpert MTB/RIF positive. Probable TB required a clinical-radiological picture compatible with TB, and initiation of anti-TB treatment by the attending clinician, but *M.tb* culture and/or Xpert MTB/RIF negative. All non-TB patients from use in the analysis given the known limitations of single sputum-based TB diagnosis in HIV-infected patients with advanced immunosuppression.

TB Diagnostic Sampling and Testing

Consultant-led hospital-based clinicians, who were not associated with the study, determined the timing and extent of TB diagnostic work-up and the commencement of anti-TB treatment. Routine hospital practice includes the collection, where possible, of two

sputum samples in patients able to expectorate. Sputum-based reference testing performed on admission and prior to treatment commencement, this was either on the same-day or prior to LF-LAM testing. The local reference laboratory processed all clinical specimens collected for TB diagnosis. Xpert MTB/RIF assay and/or culture, using MGIT 960 liquid culture system (BD Diagnostics, USA), was performed on sputum or non-sputum samples and Xpert MTB test was performed according to manufacturer's instructions (62).

Urine Sampling and LF-LAM Methodology

All patients were required to give a random urine sample (30ml) collected in a sterile container at enrolment. EMU collected the following day, first void urine of the day between 05h00 am and 07h00 am. The LF-LAM was done on spot samples at the bedside and additionally on matched frozen-thawed spot and EMU specimens. Sputum-based reference testing was performed in parallel. All samples were tested using the Alere Determine®-TB LAM Ag lateral flow assay (Alere, USA). Briefly, 60µl of urine pipetted onto the lateral flow strip-loading bay (pipettes provided with the strips). After 25-35 minutes, two independent readers blinded to the reference test results read the LF-LAM. This was done via the following procedure: test validity was confirmed by identifying the presence of a band in the positive control window; the intensity of the colour band (if any) in the patient window was read by comparison with the pre-January 2014 manufacturer-provided visual reference scale card (graded 0-5 depending on band intensity). Using the manufacturer-recommended grade 2 cut-point, a band of visual intensity \geq grade 2 in the patient window was classified as a 'positive' test while the complete absence of a band (grade 0) and faint band (grade 1) in the patient window was classified as a 'negative' test. The test reported as invalid if no control, either band identified in the patient window or if a broken / incomplete band seen in the patient window. Invalid tests repeated once, but thereafter LF-LAM was considered to have failed. Discrepancies found between two readers in binary readout of positive or negative were resolved by the third reader.

Statistical Analysis

Descriptive statistics for baseline demographic and clinical characteristics are used. Diagnostic accuracy measures included only sensitivity with the 95% confidence interval. χ^2 testing used to compare sensitivity proportion between spot and EMU samples. Predictors of an increased LF-LAM grade between random and EMU performed, using multivariate linear regression. Data was analysed, using STATA software, version 11. Evaluation of the diagnostic test parameters was done with a Diagnostic or Screening Test Evaluation 1.0, using OpenEpi (Open Source Epidemiologic Statistics for Public Health, Version 3.03a. www.OpenEpi.com). The STARD criteria was utilised for all reporting and analysis (63).

RESULTS

184 patients were enrolled, but 61 patients were excluded as they had all sputum-based TB diagnostics negative and were not given treatment (n=52) or were unable to provide a urine sample at the time of enrolment (n=9) (Figure 1). The median age (IQR) was 36 (31-41) years and 58% of patients were female. The majority of patients had advanced immunosuppression with a median (IQR) CD4 of 88 (36-209) cells/ml.

LF-LAM sensitivity in spot versus EMU

Of 184 HIV- infected adults screened, only 123 patients commenced anti-TB treatment and provided matched random and EMU samples. 33% (41/123) of included patients had culture/Xpert MTB/RIF positive and classified as definite TB and 67% (82/123) had other diagnostic features suggestive of TB, including clinical findings, radiological evidence suggestive of TB (chest x-ray, ultrasound), and clinical symptoms and commenced on treatment. These are the patients classified as probable TB. Table 1 shows the sensitivity of LF-LAM in random versus EMU samples for definite- and probable-TB groups. Overall (definite- and probable-TB combined), the sensitivity (95% CI) of EMU improved from 10% (6-17) to 30% (22-38) with EMU ($p=0.0003$ using the grade 2 cut-point. Limiting the analysis to only patients with definite TB, LF-LAM sensitivity, using the grade 2 cut-point, increased from 12% (5-24) to 39% (26-54), in random versus EMU samples, respectively ($p=0.004$); amongst probable TB, LF-LAM sensitivity increased from 10% (5-17) to 24% (16-34) ($p=0.01$).

The data was stratified by CD4 counts of >200, <200, <100 and <50 cell/mm³ to determine whether, as in other studies, CD4 cell count plays a significant role in LF-LAM grade (figure 2). There were no differences noted between random and EMU specimen sensitivity for individual CD4 strata

Predictors of increased LF-LAM positivity between spot and EMU

None of Age, Xpert MTB/RIF /liquid TB culture positivity or prior initiation of anti-TB treatment within 7 days was significant predictors of an increasing LF-LAM grade between spot and EMU specimens (Table 3).

Discussion

The WHO has recently endorsed the use of LF-LAM testing for hospitalised HIV-infected patients with suspected TB (59). In addition, our group recently published a multicentre RCT showing that a LAM-guided early TB treatment initiation strategy reduced all-cause mortality amongst this vulnerable patient group (58). However, test sensitivity remains sub-optimal, and simple strategies that could improve test sensitivity are necessary. This proof-of-principle study suggests that using EMU can increase the sensitivity of LF-LAM testing compared to random spot urine sampling. This could further improve the utility of LF-LAM for hospitalised HIV infected patients with advanced immunosuppression and possible TB.

A number of strategies already been published or are being examined to improve the sensitivity of LAM. Efforts are being made to optimise the immune-detection methods used with the development of high avidity monoclonal LAM antibodies or aptamer technologies that could improve both sensitivity and specificity (52). Differing approaches for concentrating urine samples after collection to improve sensitivity have included heating, centrifugation and/or molecular weight exclusion filtration (52). None of these approaches is the ideal answer. Designing a new LAM assay will take many years of development, while these concentration methods all require laboratory infrastructure and human resources, unavailable in many places where testing is most in need. Therefore, a natural method for antigen concentration namely EMU sampling may be a good, ease-to-implement strategy. One other recent study showed no improvement in LF-LAM sensitivity from a two urine sample strategy; however, EMU sampling was performed up to seven days after the 1st spot urine was collected, and the timing of morning sampling is not stipulated (64). This delay in sampling may have dropped LAM concentration and offset the benefit of early

morning sampling. Further studies are now required to confirm our findings, consider any impact on specificity, and most importantly to assess the impact on patient-important outcomes.

Our study did not find any factors that were associated with an increase in EMU LAM concentration, (as measured by the change in LF-LAM grade). Factors known to affect LAM concentration, such as CD4 cell count, age and culture/Xpert MTB/RIF status were not associated with an increased LF-LAM grade (65). This seems biologically plausible given that these factors would likely be unchanged between spot and EMU sampling time-points. It is likely that more immediate physiological factors, such as blood pressure, hydration status and consequent glomerular filtration rates are more important determinants of increased EMU LAM concentration. Interestingly, we did not find that anti-TB treatment initiated either prior to both spot and EMU or between spot and EMU, testing was associated with increased EMU LAM. This is against previous observation and the “treat-to-test” hypothesis, which suggests that the concentration of bacillary antigens, such as LAM, may increase immediately following the initiation of anti-TB treatment due to death and metabolism of bacilli (55). Perhaps the effect of treatment on systemic antigenemia is not as large as hypothesized and other factors such as complexing of LAM to serum proteins have larger influence on urinary LAM concentrations.

Our study had several limitations. Our study had limitations. The sample size was small and we did not collect sufficient co-variate physiological data to adequately understand which patients are likely to have an increase in urine LAM with EMU sampling. The study did not examine the effect of EMU on test specificity, and reduced specificity can negatively affect patient outcomes. However, the primary aim of this study was proof-of-principle to demonstrate that EMU could improve diagnostic sensitivity. We were concerned that specificity findings would be misleading given the misclassification bias associated with use of a single sputum TB culture/Xpert MTB/RIF for TB diagnosis in this HIV-infected population with advanced immunosuppression.

The study highlights the diagnostic implications of the timing of sample collection with EMU testing significantly improving LF-LAM sensitivity in hospitalised TB/HIV co-infected patients. In this vulnerable patient group, EMU LF-LAM, in addition to spot testing on admission, and irrespective of empiric treatment initiation, may add value. Larger studies are now required to confirm our findings and importantly, to determine if the incremental sensitivity can translate to impact on

patient important outcomes or if the delay in time required between spot and EMU sampling negates the benefits of testing.

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Table 1. Overall sensitivity of the urine LAM, using random versus EMU samples, and stratified by diagnostic category and preceding anti-TB treatment

Sensitivity 95% (CI)	Manufacturer recommended LF-LAM positive cut point (grade 2)		P-value
	Random Urine %, 95% CI, n/N	Early Morn. Urine %, 95% CI, n/N	
Definite- TB only	12 (5-24) (5/41)	39 (26-54) (16/41)	0.004
Probable TB only	9.6 (5-17) 8/82	24 (16-34) 20/82	0.01
Definite and Probable TB combined	10 (6-17) (13/123)	30 (22-38) (36/123)	0.0003
No TB Rx prior	11 (4-28) (3/27)	31 (17-50) (8/26)	0.05
TB Rx prior < 7 days	10 (2-40) (1/10)	56 (27-81) (5/9)	0.04
No TB Rx prior	15 (8-27) (8/52)	25 (15-38) (13/52)	0.1
TB Rx prior < 7 day	0 (0-11) (0/30)	23 (12-41) (7/30)	0.003
No TB Rx prior	14 (8-23) (11/79)	30 (18-38) (21/78)	0.03
TB Rx prior < 7 days	2 (0-12) (1/42)	30 (18-45) (12/40)	0.0006

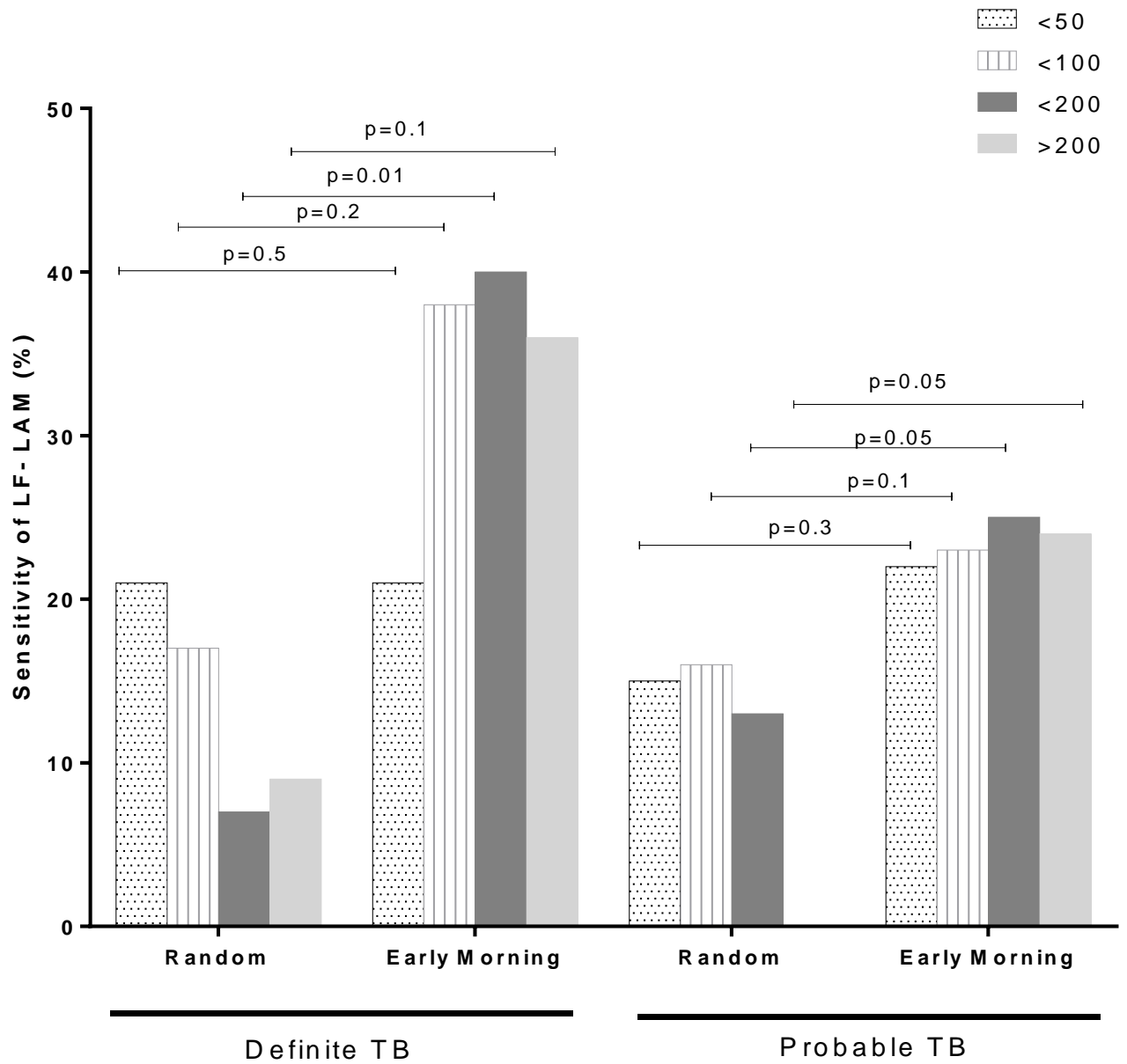


Figure 2: Overall sensitivity of the urine LAM strip test for TB diagnostic, using random versus EMU samples, and stratified by diagnostic and CD4 count

	P-value	Adjusted Odds Ratio	Lower 95% CI	Upper 95% CI
All (n=61)				
Age, years	0.32	0.967	0.906	1.03
CD4 cell count, cells/mm ³	0.92	1.000	0.996	1.005
TB culture/Xpert positive (pos/neg)	0.15	2.201	0.760	6.379
TB treatment < 7 days	0.41	1.59	0.53	4.81

Table 2. Multivariate predictors of increasing urine LF-LAM test grading between random and EMU specimens