Urine Lipoarabinomannan as Initial Markers for Active Pulmonary Tuberculosis

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Abstract: The reemergence of tuberculosis as an important public health issue and the spread of drug-resistant tuberculosis have emphasized the need for rapid diagnosis. However, the standard culture methods currently in use are quite slow. Lipoarabinomannan (LAM) is part of the Mycobacterium tuberculosis (TB) cell wall. It is released when bacteria are multiplying or dying. LAM can be detected in the urine since it is filtered from the blood in the kidneys. Urinary LAM detection is a promising approach for rapid diagnosis of active tuberculosis; in microbiologically confirmed TB patients, quantitative LAM detection results increased progressively with bacillary burden and immunosuppression. The current study was to evaluate the accuracy of the urine LAM test for the diagnosis of active pulmonary TB. Seventy two suspected TB cases were enrolled and subjected to smear microscopy, sputum mycobacterial culture and urine LAM testing using the Clearview TB ELISA kit. Fifty two patients (72.2%) had confirmed TB 42 with positive AFB smears and cultures for M. tuberculosis, and 10 (13.9%) with positive cultures but negative AFB smears. Among 52 confirmed TB cases, the LAM test was more sensitive than sputum smear microscopy “the sensitivity of LAM and sputum smear were 94.3% and 80.8% respectively”.

Key words: M. tuberculosis, Lipoarabinomannan, Human urine, Diagnosis.

INTRODUCTION

Mycobacterium tuberculosis (TB) is a major and increasing public health problem in both industrialized and developing countries. It is estimated that over one-third of the world’s population is infected, with ≥95% of all cases occurring in developing countries. The World Health Organization (WHO) estimates that in 2009, 9.4 million new cases of tuberculosis (TB) occurred and 1.7 million people died of the disease (WHO, 2010). Delayed diagnosis facilitates disease transmission, increases healthcare costs and increases the mortality and causes greater lung damage resulting in chronic disability. Mycobacterial culture, which is regarded as the diagnostic gold standard, needs 10–100 viable bacilli per ml sputum and is therefore much more sensitive but requires a maximum incubation time of 6–8 weeks (Dheda et al., 2009).

The development of new inexpensive, rapid and field adapted methods for its diagnosis is urgently needed. Sputum culture, which is still the reference method for the diagnosis of pulmonary TB, is cumbersome and time-consuming. Microscopy of direct smears for acid-fast bacilli as recommended by WHO for developing countries is the most commonly used method for diagnosis of TB (Pai et al., 2006). Although acid-fast staining of bacilli in sputum smears is a simple and relatively fast means for detection of active TB, sensitivity is compromised because greater than 10⁴ bacilli per ml of sputum are required for reliable detection (Pai et al., 2006). Thus, approximately one-half of the cases of active pulmonary TB are smear-negative, the failure rate being substantially greater in children, the elderly, and patients with acquired immunodeficiency syndrome (AIDS) (Patel et al., 2009 and Lawn et al., 2009).

Newer technologies such as the T cell assays are not useful as rule-in tests for the diagnosis of active TB in adults (Dheda et al., 2009), and molecular assays are not widely available in high burden countries (Chan et al.,2000). A new diagnostic capable of replacing sputum smear could save 400,000 lives annually (Daley et al., 2009).

Detection of mycobacterial lipoarabinomannan (LAM), a 17.5 kD heat-stable carbohydrate antigen with glycosidic linkages for which no human degrading glycosidases are known (Hunter et al., 1986; Roach et al., 1993) is a component of the outer cell wall of mycobacteria, was originally described using serum, (Hamasur et al., 2001) but this test was limited by immune complex formation (Patel et al., 2009). It assumed that in active mycobacterial disease LAM filtered through the kidneys and occurs in urine in antigenically intact form and can...
be detected in the urine as potential same day diagnostic test for tuberculosis. This test option is attractive because urine is a sterile and easily obtainable biological fluid that can be assayed even in sputum scarce patients. Moreover, the assay has potential to be applied to other biological fluids e.g. cerebro-spinal fluid and pleural fluid (Patel et al., 2009), and a user-friendly dipstick prototype of the test has been developed and is currently being validated as a POC test (Dheda et al., 2010). Moreover, urinary specimens do not carry the risks inherent to needles and blood-based laboratory work. Furthermore, if the urine specimens are boiled before handling, there is no need for BSL3 facilities. In serum, detection of LAM is complicated by immune complex formation while its detection in sputum is possible only for patients with pulmonary TB. LAM excretion in urine, however, is expected to be independent of anatomical location of the mycobacterial infection. The urinary LAM-ELISA can provide a tool for the diagnosis of pulmonary, as well as extra pulmonary of mycobacterial infection (Dheda et al., 2010).

A case-control study demonstrated promising diagnostic performance of LAM in urine (Dheda et al., 2009; Lawn et al., 2009) that was later supported by a study in Tanzania (Mutetwa et al., 2009). Based on these early data, a prototype urinary LAM detection test was produced by Chemogen (South Portland, ME, USA). A commercial version of this test is now marketed as Clearview® TB enzymelinked immunosorbent assay (ELISA; Inverness Medical Innovations Inc, Scarborough, ME, USA). LAM is specific to mycobacteria (Reither et al., 2009) is released by metabolically active bacteria (Shah et al., 2009) and is filtered by the kidney (Daley et al., 2009). LAM excreted by patients with active TB may be present in urine in variable amounts, between 0.5 and several hundred ng/ml (Wilson et al., 2006).

A urine test could facilitate TB diagnosis in patients in whom sputum is uninformative or not obtainable, and lacks the infection control risks associated with sputum production or blood collection. Finally, urine LAM detection may be amenable to simple and inexpensive. The Clearview™ TB ELISA assay (Inverness Medical Innovations, Waltham, Massachusetts, USA) is a direct antigen sandwich immunoassay in a 96-well plate format (Maunank et al., 2009).

The aim of this study is to evaluate the accuracy of the urine LAM assay in diagnosis of the active pulmonary TB in patients admitted to the hospital with a presumptive diagnosis of TB.

Patients and Methods:

Between August 2009 and November 2011, 72 patients presenting with pulmonary tuberculosis symptoms “suspected TB” were selected from the hospital. Study-directed testing included smear microscopy, sputum mycobacterial culture and urine LAM testing using the Clearview TB ELISA kit. The suspected cases of pulmonary TB were submitted for sputum specimens collection each morning on three consecutive days which were used for smear microscopy and TB culture, all patients also submitted one fresh urine specimen (≥5 ml) in a sterile container at the first study visit, prior to start TB treatment.

Smear Microscopy:

A Ziehl-Neelsen smear was prepared according to (Gerhardt et al., 1994) then examined under x100 magnification using a light microscope (Nikon ECLIPSE50i, Japan). All smears were graded as (negative: no AFB (acid fast bacilli) per 300 fields; 1+: 1 to 9 AFB per 100 fields, 2+: 10 to 99 AFB per 100 fields, and 3+: 1 to 10 AFB per field in at least 50 fields).

Mycobacterial Cultures:

Sputum specimens were decontaminated with N-acetyl-L-cysteine / sodium hydroxide (BBL™ MycoPrep™ BD Diagnostics Systems, Sparks, MD), after centrifugation at 3,000 rpm for 15 min, the pellet was suspended in buffer. A 0.5-ml portion of processed sputum sediment was cultured using the BACTEC MGIT 960 system (BD Diagnostics Systems, Sparks, MD) (Maunank et al., 2009).

Enzyme Linked-Immune sorbent Using the Clearview™ TB ELISA kit:

Urine specimens were transported to the laboratory; an aliquot of each urine specimen was heated to 100°C for 30 minutes, then centrifuged at 10,000 rpm for 15 minutes. The supernatant was stored frozen at −20°C within 24 hours of collection. Testing and interpretation were performed according to the manufacturer’s instructions; 100 μl of processed urine was added in duplicate to appropriate wells, positive and negative controls provided with the kit were run in duplicate in appropriate wells and the plates were sealed using film provided in the kit. The plates were incubated at 24°C for 1 h, then decanted into a collection basin, and tapped firmly over a paper towel. Wells were manually washed four times with 300 μl of wash buffer and decanted into a collection basin. LAM-specific horseradish peroxidase conjugated polyclonal antibody (100 μl) was added and sealed plates were incubated at 24°C for 1 h, then washed four more times. Tetramethylbenzidine single component chromogenic substrate (100 μl) was added and plates were incubated at 24°C for 15 min. The reaction was stopped with 100 μl of 1M H2SO4 (in-house) with gentle shaking, to achieve a yellow colour. Optical density (OD) was read immediately at 450 nm. As recommended by the manufacturer, the sample was reported as
positive if OD was at least 0.1 above the signal of the negative control. If both replicates of patient samples failed to agree or were not both within 15% of the mean OD value, the test was repeated. If the negative control was not between 0.15 and 0.25 absorbance units, or if the positive control was not between 0.3 and 0.5 absorbance units above background, the test was repeated (Maunank et al., 2009, Redwan and El-Awady, 2002).

**Results:**

The study included 72 patients; 50 (69.4%) were males. Fifty-two patients (72.2%) had confirmed TB (42 with positive AFB smears and cultures for *M. tuberculosis*, and 10 (13.9%) with positive cultures but negative AFB smears) (Table 1).

**LAM Test:**

Thirty nine (54%) were positive by both the LAM test and smear microscopy, 20 (27.8%) were negative by both assays, 10 (13.9%) were positive by the LAM test alone, and 3 (4.2%) were positive by smear microscopy alone (Table 2). 49 (68.1%) were positive by both the LAM test and cultures for *M. tuberculosis*, 20 (27.8%) were negative by both assays, and 3 (4.2%) were positive by *M. tuberculosis* cultures alone (Table 3).

Among 52 confirmed TB cases, the LAM test was more sensitive than sputum smear microscopy “the sensitivity of LAM and sputum smear were 94.3% and 80.8% respectively”.

**Table 1: Confirmed TB cases.**

<table>
<thead>
<tr>
<th>AFB smear</th>
<th>Cultures for <em>M. tuberculosis</em></th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
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<tr>
<td>Positive</td>
<td>42</td>
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</tr>
<tr>
<td>Negative</td>
<td>10</td>
<td>20</td>
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<tr>
<td>Total</td>
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<td>20</td>
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</table>

**Table 2: LAM test results in comparison to sputum smear.**

<table>
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<tr>
<th>AFB Smear</th>
<th>LAM</th>
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<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>39</td>
<td>03</td>
</tr>
<tr>
<td>Negative</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>49</td>
<td>23</td>
</tr>
</tbody>
</table>

**Table 3: LAM test results in comparison to sputum culture.**

<table>
<thead>
<tr>
<th>Cultures of <em>M. tuberculosis</em></th>
<th>LAM</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
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<tr>
<td>Positive</td>
<td>49</td>
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<tr>
<td>Negative</td>
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<td>Total</td>
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**Discussion:**

Pulmonary TB (PTB) was defined as the presence of one or more sputa positive for AFB by microscopy and/or positive for *M. tuberculosis* by culture. In our study; ten patients (19.2%) were positive sputum cultures but negative AFB smears (AFB sensitivity 80.8%) which were explained by many authors before. Corper (1928) estimated that 100 000 acid-fast bacilli per milliliter of sputum was the minimum concentration that could be detected by direct microscopy. However Tazir et al. (1979) suggested that 90-96% of specimens containing between 30.000 and 50.000 acid-fast bacilli per milliliter and 50-58% of those containing between 2.000 and 5.000 bacilli per milliliter give positive Ziehl-Neelsen stained smears (Cowan and Steel's manual for the identification of medical bacteria). WHO (2010) stated that direct smear microscopy is relatively insensitive as at least 5.000 bacilli per milliliter of sputum are required for direct microscopy to be positive. An important attribute of the LAM test was its ability to detect 10 (19.2%) of confirmed TB cases not detected by sputum smear microscopy with sensitivity (94.3%), this is a relatively large incremental yield compared to smear microscopy. This suggests that the LAM assay and smear microscopy may detect different groups of TB patients, and might be best used in combination, as was reported by Shah and colleagues (Jessica et al., 2011).

Current study demonstrated that the LAM sensitivity and specificity were (94.3% and 100%, respectively); there were many studies that discussed the LAM assay in urine; Boehme and colleagues used a prior version of the existing urine LAM assay (Chemogen, South Portland, Maine) to evaluate 231 TB suspects (69% HIV-positive) and 103 healthy controls in Tanzania (Boehme et al., 2005). Sensitivity was 80.3% among individuals with *M. tuberculosis* isolated from sputum culture; specificity was 99% in healthy controls. Maunank et al. (2009) used unprocessed fresh urine whereas the present study used concentrated, frozen urine. Corbett and colleagues recently evaluated accuracy of the Clearview™ TB ELISA test in TB patients and suspects in Harare, and found that the sensitivity was 52% among HIV-infected, TB culture-positive individuals (Mutetwa et al., 2009). Maunank et al. (2009) revealed in unconfirmed TB patients that the LAM test was more sensitive and significant than sputum smear microscopy (42%, 82/193, p<0.001) and detected 56% (62/111) of those who were
sputum smear-negative LAM. In seven studies that assessed test accuracy in microbiologically confirmed cases only, estimates of sensitivity ranged from 13% to 93%, while specificity ranged from 87% to 99%. In five studies that assessed accuracy in clinical and confirmed TB cases, sensitivity ranged from 8% to 80%, while specificity ranged from 88% to 99% (Jessica et al., 2011).

Conclusion:
The benefits of using a non-invasive, easily collected specimen such as urine would be greatly appreciated in the diagnosis of TB as currently confirmed. While the urine LAM urine assay test is unlikely to stand alone for definitive TB diagnostic testing, its usage is attractive as a rapid diagnostic modality that complements smear microscopy and has many characteristics which make it a potentially useful rule-in TB diagnostic.

REFERENCES


