Modification of Clearview® TB ELISA for tuberculosis patients without HIV

Running title: Detection of LAM from concentrated urine

Laura Savolainen, a Anu Kantele, a,b,c Bettina Sandboge, a Marita Sirén, a Heikki Valleala, d

Riitta Tuompo, d Liana Pusa, e Riitta Erkinjuntti-Pekkanen, f,g Aija Knuuttila, h Cheng-Lung Ku, i Chih-Yu Chi, j Tuula Vasankari k and Tamara Tuuminen a,l

a Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, Helsinki, Finland
b Division of Infectious Diseases, Department of Medicine, Helsinki University Central Hospital, Helsinki, Finland
c Institute of Clinical Medicine, University of Helsinki, Helsinki, Finland
d Division of Rheumatology, Department of Medicine, Helsinki University Central Hospital, Helsinki, Finland
e Länsi-Uusimaa Hospital, Tammisaari, Finland
f Unit of Medicine and Clinical Research, Pulmonary Division, University of Eastern Finland
g Division of Respiratory Medicine, Department of Medicine, Kuopio University Hospital, Kuopio, Finland
h Heart- and Lung Center, Helsinki University Central Hospital, Helsinki, Finland
i Graduate Institute of Clinical Medical Science, Chang Gung University, Kwei-Shan Tao-Yuan, Taiwan
j Division of Infectious Diseases, Department of Internal Medicine, China Medical University Hospital, Taichung, Taiwan
k Finnish Lung Health Association (FILHA), Helsinki, Finland
l Eastern Finland Laboratory Centre Joint Authority Enterprise, Mikkeli, Finland
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Correspondent footnote: laura.e.savolainen@helsinki.fi
Diagnosis of active tuberculosis by detection of urinary lipoarabinomannan (uLAM) from *Mycobacterium tuberculosis* is an attractive approach. Concentrating urine 100-fold allowed quantitation of uLAM at levels equaling to picograms/ml of non-concentrated urine. The approach of concentrating urine 100-fold improved clinical sensitivity of the Clearview® TB ELISA from 7% to 57%, yet impaired its specificity from 97% to 89%.
Measuring microbial antigens excreted into urine offers an attractive approach to diagnose acute infections (1-3). While the diagnostics of tuberculosis (TB) is challenging, an appealing approach is to detect urinary lipoarabinomannan (uLAM), the major structural component of the outer cell wall, shed into the environment by replicating, metabolically active or degrading mycobacteria (4-6). Several publications have reported the use of the Clearview® TB ELISA (Inverness Medical Innovations, Bedford, UK) (7-12) or MTB LAM ELISA Test® (Chemogen, Portland, USA) (13-17) to detect uLAM. The assay has almost invariably been found to have better sensitivity for cases with advanced HIV infection than without HIV (7, 8, 11, 12, 16, 17). This has been explained by the progressively increasing bacillary burden in TB+/HIV+ patients after the profound loss of CD4+ T cells, and the inability to restrict mycobacterial growth, which results in heavy antigenemia – and excretion of higher amounts of uLAM (8, 11, 12, 15).

The present study was carried out to (a) examine whether concentrating urine will improve analytical sensitivity of the Clearview® TB ELISA in TB+/HIV- patients; (b) estimate the quantities of LAM excreted; (c) look into the factors affecting analytical performance; and (d) investigate the correlation of LAM detection from concentrated vs. non-concentrated urine with sputum staining.

Mid-stream urine samples were collected in Finland and Taiwan from adult patients with active pulmonary (P^A^1-TB, n = 28; P^A^2-TB, n = 17) or extra-pulmonary TB (E^P^1-TB, n = 7; E^P^2-TB = 3), miliary (n = 2), latent (LTBI, n = 15) or treated TB infection (n = 4), from disease control groups (n = 60) and healthy volunteers (n = 101). The clinical and demographic details on enrolled groups are presented in the supplements (S1-S2); the regents and the procedure are described in S3. Ethical clearances: DnoNo 47/180/2009; 149/2010; 105/2010 and DMR-99-IRB-075-2.
When optimizing the assay, we found that LAM dissolved in urine produced higher optical densities (ODs) than that dissolved in water, and a wide range of pH (> 3) was tolerable without deterioration. The calibration curves were prepared as described in S3; the effect of concentration on the ODs is shown in S4. The theoretical analytical sensitivity (8 replicates) was 320 and 15 pg/ml for the non-concentrated and concentrated urine, respectively.

The urine samples were analyzed in both non-concentrated and 100-fold concentrated form. As shown Fig 1A and B, the ODs were higher and the dynamic range wider for the 100-fold concentrated (OD range 0.132-3.060) vs. the non-concentrated (0.132-0.395) samples. Although a statistically significant difference was reached for patient groups both with the concentration ($p < 0.001$) and the original method ($p < 0.001$), practical discrimination between the groups seemed possible only with the modified approach.

Generally, assay imprecision tends to be higher at low OD values, which leads to inconsistent and inaccurate interpretations. Using the modified method with calibration curves constructed for each run (e.g. Fig. 1C) and ROC analysis (S5), the cut-off level was estimated at 1.1 ng/ml (S6). In these settings uLAM was detectable in 16/28 (57%) of P$^{F}$-TB patients (Fig. 1D). When taking into account the 100-fold concentration coefficient, the estimated range of excreted uLAM in native samples of the P$^{F}$-TB group proved to be 0 - 170 pg/ml; in the EP$^{F}$-TB group uLAM excretion was at maximum 14 pg/ml whereas in a patient with miliary TB uLAM was excreted at 166 pg/ml. In a sample from another patient with miliary TB, uLAM was still detectable at a concentration of 24 pg/ml after three-months specific therapy.

Overall, the concentration approach improved the sensitivity of the assay from 7% to 57%, but decreased its specificity from 97% to 89% (S6). An analysis of the false positivity rate showed that concentration did not always augment false reactivity. Interestingly, in some samples false positivity disappeared after concentration, but occurred...
at the same time in some others; the ODs were however close to the cut-off. The majority
12/15 (80%) of samples in the UTI group gave a false positive result when analyzed as a 100-
fold concentrate, while only one sample without concentration proved positive (7%) (S6-S7).
On the other hand, spiking urine with *E. coli*, *E. faecalis* and *C. albicans* at $10^4 - 10^6$ cfu/ml
did not cause false positivity (S7). With *Corynebacterium sp.* it did, yet in a dose-dependent
manner (S7). Spiking with *M. tuberculosis* produced high ODs already at 10 cfu/ml (S7).
Samples from pneumonia groups PNC and NonPNC showed false positive results in 2/7
(29%), 3/7 (43%), and 0/7 (0%), 1/7 (14%) cases when analyzed 75-fold and native,
respectively (S6-S7). D-mannose and L-arabinose at concentrations of 1 mg/ml each had no
effect on the results. uLAM was not detectable in a sample from a patient with pulmonary *M.
avium* infection, but in a modified method uLAM was detectable at 12 pg/ml (non-
concentrated). It appears probable that polyclonal antibodies of the assay cannot discriminate
AraLAM from ManLAM of the cell wall structures of non-tuberculous and pathogenic
mycobacteria, respectively (18).

Compared with sputum staining, uLAM detection identified some but not all
patients, thus the techniques are supplemental (Table 1). There were only two cases with
simultaneously positive uLAM (non-concentrated) and sputum smear staining, but more
samples became so when the modified method was used.

uLAM excretion kinetics could be exploited in treatment monitoring, as they
are utilized for some other infections (2, 19, 20). Samples from a patient with PFT-TB were
analyzed at 11, 14, and 15 days, and 9 months later. When used in a concentrated form, the
acute samples were clearly above the cut-off, but the convalescent sample was negative. By
contrast, when the samples were tested intact, uLAM was not detectable at any time point
(S8).
The reported sensitivity and specificity for the Clearview™ TB ELISA method were 13% - 93% and 87% - 99%, respectively (4-6). As far as we are aware, this is the first study to demonstrate that urine concentration, a simple pre-analytical step, can improve the detection of low amounts of uLAM in TB+/HIV- patients and raise clinical sensitivity, yet, as a trade-off, somewhat decreases specificity. Impairment of specificity was most evident in the UTI group. These results were consistent with earlier findings (13), but false positivity might not be caused by cross-reactivity with bacterial components per se. Rather, non-specificity could be attributed to inflammation, an issue to be studied further. The specificity of polyclonal antibodies in the Clearview™ TB ELISA is not reported. LAM molecules are not confined to mycobacteria (18), and therefore, the antibodies may recognize LAM-like structures from actinomycetes. Our data corroborated earlier findings that small amounts of Corynebacterium sp belonging to microbiota of mucous membranes can potentially be hazardous. Antigens from several species of fungi were reported to produce cross-reactivity (8). In the present study Candida albicans, opportunistic yeast found often in female genitourinary tract, had no such effect.

Qualitative analysis only allows a dichotomous distribution to positives and negatives. It has been reported that uLAM is excreted in variable concentrations between 100 pg/ml and several hundred ng/ml (9, 13, 15, 21). If 100 pg/ml were to be a clinically relevant concentration, the cut-off of the method should be well below this value. However, using the Clearview™ TB ELISA without the concentrating step, 100 pg/ml is not distinguishable from the background values (see S4). The analytical sensitivity of an in-house EIA was 1 ng/ml of 50-fold concentrated LAM, which corresponds to 20 pg/ml of the untreated urine (22). This method, with a 450 pg/ml as a cut-off, was evaluated later on Ethiopian TB patients, and attained clinical sensitivity and specificity 74% and 90%, respectively (21); the HIV status of the patients was not reported, however. In another study, only a modest improvement (from
was reached with the MTB LAM ELISA Test® when the samples from HIV+/TB+ patients were concentrated, however the concentration coefficient was not reported (15). In a study where the Clearview® TB ELISA was used as instructed, the median concentrations of excreted uLAM were 1.25 (IQR 0.2 - 7.1) ng/ml and 0.1 (IQR 0 - 0.5) ng/ml in patients with and without immune reconstitution inflammatory syndrome (IRIS), respectively (9). In our opinion, if the manufacturer’s instructions are followed, uLAM in 0.1 ng/ml can hardly be distinguished from the zero-calibrator (S4-B).

It has recently been hypothesized that the negative results obtained with the Clearview® TB ELISA for some TB patients could be explained by the formation of immune complexes between the excreted uLAM and anti-LAM immunoglobulins (11). The molecular size of these complexes may be too big for these molecules to pass through the glomerular basement membrane. This hypothesis seems far too theoretical, because anti-LAM antibodies might be found in the circulation of all TB patients (23). As TB is, in fact, a chronic condition, the immune system is continuously being sensitized to alien antigens. We incline to believe that negative results are more likely to be attributed to low assay sensitivity.

Furthermore, when the excretion of M. tuberculosis DNA into the urine of TB+/HIV+ patients was tested with the Xpert® MTB/RIF assay (11), a positive correlation with the Clearview® TB ELISA was found. This may mean that mycobacteriuria, which might occur due to disseminated or urogenital TB of HIV+ individuals, accounts for the better sensitivity of the Clearview® TB ELISA for samples from these patients.

To conclude, the present study is the first to show that a pre-analytic 100-fold concentrating step increases significantly the sensitivity of the Clearview® TB ELISA in the TB+HIV- group. Our data suggest that the detection of uLAM from a concentrated sample may serve as a viable tool for TB diagnostics in various types of TB patients, yet this method still needs to be refined.
REFERENCES


SB. 2001. Rapid diagnosis of tuberculosis by detection of mycobacterial lipoarabinomannan

Integration of microscopy and serodiagnostic tests to screen for active tuberculosis. Int. J.
Tuberc. Lung Dis. 9:1120-1126.
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Conflict of interest statement

The authors declare that they have no financial relationship with any commercial entity that has any interest in the subject of this manuscript.

Authors contributions

Design of the study: TT, LS, BS; applications for ethical permission: TV, AK, TT, BS, C-LK; collection of clinical samples: AK, LS, TV, LP, REP, HV, RT, TT, AK, C-YC.

LS, BS and MS performed the study: TT and LS interpreted the results and drafted the paper.

All authors contributed to critically revising the manuscript.
Figure legends

FIG 1 Analysis of urine samples from patient groups and healthy volunteers. ODs of non-concentrated (A) and concentrated (B) samples from groups Healthy (n = 101), P^F -TB (n = 28), EP^F -TB (n = 7). (C) Calibration curve of uLAM, (range 0 - 20 ng/ml, non-concentrated). (D) uLAM concentrations estimated from the calibration curve. Healthy (n = 101), P^F -TB (n = 28), EP^F -TB (n = 7) and LTBI (n = 15). Horizontal bars show the mean concentration. The cut-off (1.1 ng/ml) is presented with a dashed line.
Table 1 Comparison of a positive uLAM detection and a positive sputum staining.

<table>
<thead>
<tr>
<th>100-fold concentrated urine</th>
<th>AFB*+ n (%)</th>
<th>AFB- n (%)</th>
<th>Total LAM n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAM+</td>
<td>16 (57)</td>
<td>1 (4)</td>
<td>17 (61)</td>
</tr>
<tr>
<td>LAM-</td>
<td>8 (29)</td>
<td>3 (11)</td>
<td>11 (39)</td>
</tr>
<tr>
<td>Total AFB</td>
<td>24 (86)</td>
<td>4 (14)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Non-concentrated urine</th>
<th>AFB+ n (%)</th>
<th>AFB- n (%)</th>
<th>Total LAM n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAM+</td>
<td>2 (7)</td>
<td>0 (0)</td>
<td>2 (7)</td>
</tr>
<tr>
<td>LAM-</td>
<td>22 (79)</td>
<td>4 (14)</td>
<td>26 (93)</td>
</tr>
<tr>
<td>Total AFB</td>
<td>24 (86)</td>
<td>4 (14)</td>
<td></td>
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* Acid fast bacilli