A Novel Urine Method for the Diagnosis of Active Tuberculosis by Immunoassay for the Detection of ESAT-6 Using Hydrogel Nanoparticles in HIV Patients

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Abstract

Background: In patients with HIV, conventional tests are of low sensitivity; therefore, a new diagnostic test with hydrogel nanoparticles with reactive blue dye is proposed, which allows the capture, conservation and concentration of ESAT-6 in urine. NIPAs are copolymers that capture low molecular weight proteins and protect against enzymatic degradation. Using an immunoassay, it is possible to detect and quantify ESAT-6 in urine as a possible marker of active TB. Design/Methods: Study in Lima, Peru, HIV+ participants, ≥18 years with and without tuberculosis (TB). Smear microscopy, culture in solid medium and urine immunoassay were performed. The reference was the diagnosis of TB by radiological or clinical microbiological criteria (indication for TB treatment). There were 2 preanalytical processes: untreated and treated urine (centrifuged, heated), then incubation with NIPAm. After washing, elution, sonication, heat and centrifugation, the final eluate was obtained. This was placed on nitrocellulose membranes, which by means of fixation and incubation processes with anti-ESAT-6 and anti-IgG antibodies, revelation with C-DiGit® Blot Scanner and FluorChem R FR0001. Calibration curves were included in the membranes, density was measured using Image J software. ROC curves, sensitivity and specificity were obtained. Results: The result by groups was HIV+ patient: ROC: 0.75, cut-off point ≥24.06 ng/ml, sensitivity 76.32%, specificity 68.89%, patients ≤200 cells CD4 mm³/ml, ROC: 0.78, cutoff point ≥26.20 ng/ml, sensitivity 75.86%, specificity 71.88%, pa-
patients >200 CD4 mm$^3$/ml, ROC: 0.73, cutoff point ≥24.6 CD4 mm$^3$/ml, sensitivity 73.68%, specificity 73.68%. **Conclusions:** The ESAT-6 detection assay using NIPAm was effective, with higher rates in patients with ≤200 CD4 cells/mm$^3$, the test being more sensitive than smear and culture, but less specific.

**Keywords**

Tuberculosis, HIV+, Hydrogel Nanoparticles and ESAT-6

1. Introduction

Currently, tuberculosis (TB) and human immunodeficiency virus (HIV) infection are two of the leading causes of infectious mortality worldwide [1]. Approximately 25% of the world’s population are infected with TB and at possible risk of active disease. In 2018, the World Health Organization (WHO) reported 10 million new TB cases, of which 8.6% also had HIV infection [2], approximately 251,000 deaths. In Peru, it is the main cause of death in the HIV patients TB [3] [4] [5].

Patients HIV positive are highly susceptible to tuberculosis infection, due to their compromised immune system, and therefore low immune response, which does not form caverns, therefore, his patients had a decreased proliferation of bacilli, that difficult the conventional test like direct bacilli observation, culture and nucleic acid amplification [6] [7] [8]. This is the main reason for late diagnosis and treatment initiation, increasing the probability of M. Tuberculosis spread to other organs.

Currently, in low and middle countries the diagnosis of TB in HIV population is based on smear ZN staining, a test with low sensitivity 28%, cultures (Ogawa, MODS and Bactec) have sensitivity greater than 68%, however takes a long incubation period and need high biosecurity measures [9] [10] [11] [12]. Molecular tests such as Xpert MTB/RIF 79%, genotype 30% are very effective, but high cost, also, are aimed more to determine drug sensitivity than diagnosis. Therefore, its implementation and use in low complexity laboratories is not very feasible [13] [14]. Radiology diagnosis is not very effective in HIV patients because they don’t present the conventional radiologic patrons.

Several studies are searching active TB markers in other samples than sputum, focus on M. tuberculosis antigens detection in urine, due to its easy sample collection, less risk of aerosols for laboratory personnel and lower cost per application and use in a low complexity laboratory. However, the enzymatic degradation of antigens in urine doesn’t allow the antigen detection [15].

Other tests have been developed, such as the lipoarabinomannan detection (LAM) in urine by immunochromatography, which is applied in patients with HIV infection with <200 CD4 mm$^3$ cells with a sensitivity of 4.0% and up to 66.7% in <50 CD4 mm$^3$ cells [12]. Due to this, its use is diminished and mainly
applicable to diagnosis disseminated TB, a very advanced stage of tuberculosis infection [7] [8] [16] [17]. Thus, it is necessary to develop diagnostic tests that aren’t based on the detection or isolation off bacillus in sputum, but on the detection of Mycobacterium antigens that are present in an easily obtained sample such as urine. Esat-6 antigen has been investigated to have greater sensitivity and specificity [7] [18] [19] [20]. However, its use in a diagnostic test has not yet been accomplished.

Hydrogel nanoparticles (NIPAm) have been applied, which are three-dimensional hydrophilic copolymers of N-isopropylacrylamide (pNIPAm) [2] that, by size exclusion and affinity chromatography in solution, capture low molecular weight proteins, concentrate and protect of endogenous enzymatic degradation. This method of using nanoparticles to capture antigens in infectious diseases has been used as congenital Chagas disease, showing promising results. In vitro studies carried out by George Mason University (USA) report a 50-fold increase in the detection capacity of ESAT-6, CFP10 and LAM by SDS PAGE compared to the normal value [21] [22] [23] [24] [25]. Hydrogel nanoparticles made on a reactive blue base (NIPAm/rb) have been shown to be the most efficient and best captured ESAT-6 antigens in Western blot analysis.

Due to the need to develop new simple, fast and easy-to-implement diagnostic tests, with greater sensitivity and specificity a new method is with the use of hydrogel nanoparticles for the determination of ESAT antigen-6. Profiling as one of the easiest to apply due to its ease in obtaining the sample and the possible greater sensitivity than other tests.

For these reasons, the main objective of this study is to determine a diagnostic method for active TB in patients with HIV co-infection, through the detection of ESAT-6.

2. Materials and Methods

2.1. Study Design and Population

Experimental study of the diagnostic test, carried out in Lima, participants from Dos de Mayo National Hospital and 7 peripheral centers belonging to 4 study groups: group 1 (TB+, HIV+), 2 (TB−, HIV+), 3 (TB+, HIV−) and 4 (TB−, HIV−), ≥18 years and capable of produce a sputum sample. TB+ status was defined as active TB from any case with less than 3 days of TB treatment and HIV + status was by documented HIV infection. Enrollment was carried out in 2 years (02/2017-02/2019). Sputum samples were collected and a random non-sterile urine sample with minimum volume 90 ml. All sputums had smear and culture in Ogawa; MODS in some cases (When there was a enough sputum sample volume); immunoassay for ESAT-6 with NIPAm in urine and rapid HIV test (where applicable). Likewise, a socio-economic questionnaire, TB contact, medical history, concomitant treatment, as well as signs and symptoms of TB were applied. Drug susceptibility testing of standard care was recorded.
The diagnosis of TB was made by 3 criteria: microbiological, positivity to smear, Ogawa or MODS culture, hain testin sputum and other samples; radiological, patterns compatible with active tuberculosis infection and indication of immediate; and clinical TB treatment, determined by signs and symptoms compatible and indication of immediate TB treatment. HIV+ patients were followed up for 1 year, and those who later presented TB were group reassigned. Follow-up was carried out by patient self-report if they had active tuberculosis in the previous year.

The samples were processed in two laboratories: Center for Applied Proteomics and Molecular Medicine, at George Mason University (U. Mason) and at the Biochemistry and Informatics Laboratory, Research and Development Laboratories, Universidad Peruana Cayetano Heredia (UPCH). Test standardization processes were carried out in both laboratory.

2.2. Experiment

A urine sample was collected, transported in 2°C - 8°C. There were 2 preanalytical procedures for treated and untreated urine 1) Untreated urine, directly incubated with NIPAm (45/1), incubated at room temperature for 30 minutes and centrifuged at 4000 g for 20 minutes, the pellet resuspended in 1 ml of urine, aliquoted and stored at −80°C. 2). Treated urine, immediately frozen at −80°C, preserve and in laboratory conditions heated to 100°C in drie heat for 10 minutes, centrifuged at 16,000 rpm for 15 minutes. A 1 ml aliquot of supernatant is recovered, and incubated with 200 μl of NIPAm at room temperature and subsequently, centrifuged at 16,000 rpm/min with the aim of obtaining the complex ESAT-6 + Nipam pellets. In the untreated urine samples, the Nipam vol was recorded to adjust the ESAT-6 concentration, this factor allowed the samples to be compared with the calibration curve.

Calibration curve, included in each immunoassay, with concentrations of recombinant antigen ESAT-6 (abcam, ab124574) and a negative control splashed into 1 ml of urine were included. In treated urine, concentrations of 100 ng/ml, 50 ng/ml, 25 ng/ml and 0 ng/ml were included and in untreated urine, 100 ng/ml, 40 ng/ml, 10 ng/ml and 0 ng/ml. Depending on the type of preanalytical (treated and untreated urine), the curve passes the same preanalytical procedures.

Elution, the pellets (Nipam + low molecular weight proteins) were washed 3 times with H₂O Milli-Q, centrifuged at 16,000 rpm for 15 minutes. The pellet was eluted with 50 μl of Novex™ Tris-Glycine SDS Sample Buffer (2×) (5% 2-Mercaptoethanol, CAS Number 60-24-2), subsequent sonication for 30 minutes at room temperature, heated to 100°C for 5 minutes and centrifuged at 16,000 rpm/min.

The supernatant passed the HiPPR™ Detergent Removal Spin Column Kit columns according to the manufacturer’s instructions, obtaining the detergent-free eluate for immunoassay.

Immunoassay, an immunoblotting membrane (IMMUN BLOT PVDF MEM,
1620176, Biorad) was hydrated with 1 alcohol bath and 3 water for 1 minute each. To the hydrated membrane, the calibration curve and the samples were included in spots, two rounds of 1 μl each.

Incubation was performed by rotation at room temperature (1 - 3 hours) and in refrigeration for long periods (4 - 12 hours). The times and dilutions varied according to the procedure of each laboratory.

The membrane was incubated with blocking buffer (phosphate buffer saline (PBS), 0.2% I-block), incubation time 1.5 - 12 hours (U. Mason) or 2 hours (UPCH). Subsequent incubation with anti-ESAT-6 monoclonal antibody (abcam, ab26246) diluted in blocking buffer, dilution 1/250, for 1.5 - 12 hours (U.Mason) or 1/1000, for 3 hours (UPCH). 3 washes with PBS-T (PBS, 0.1% tween) for 15 minutes each.

The next incubation is with horseradish peroxidase-labeled goat polyclonal secondary antibody (Invitrogen A16084), dilution 1/5000 with incubation time for 2 hours (U. Mason) and for 1 hour (UPCH). Consecutively, 3 washes with PBS-T of 15 minutes each were carried out.

Then, the development is with radish peroxidase (HRP) substrate of enhanced chemiluminescence based on luminol (ECL) for 5 minutes away from light.

Two chemiluminescence systems were used, in Mason, FluorChem R FR0001; UPCH, C Digit Blot Scanner. Photographs were taken of the membrane without a filter with a light exposure of 30 seconds and 3 minutes, the photograph with the best resolution, the lowest background, and the highest reactivity in the positive controls was chosen.

Reveled, spot intensity was measured with Image J NIH software. A relationship between calibration curve and their image intensity was established. Using this equation, the simple concentración was calculated.

In some membranes with an unclear and therefore invalid calibration curve, the equation of a valid standard curve of a membrane performed on the same day (same eluates of the calibration curve) was used.

2.3. Analysis Plan

Statistical software Stata 13 was used. The relationship equation between the spots densities and the curve concentration was performed by linear regression. ROC (Receiver operating characteristic) curves were used to determine sensitivity and specificity of different cut-off points for the concentration of ESAT-6 in urine for the diagnosis of TB in the HIV population, having as a reference test, positivity at least one of the microbiological criteria, clinical or radiological. Comparisons were made between diagnostic and reference tests, and I also made comparisons between each test. Likewise, subgroups of patients were analyzed regarding pulmonary and extrapulmonary TB status, as well as CD4 level, and the sensitivity and specificity in each subgroup were calculated. The statistical power of the sample size was 89.90% with a significance level of 0.05, using an independent group comparison and expected sensitivity of the test of 60% com-
pared to the 30% smear test.

2.4. Ethical Considerations

The protocol was approved by the Ethics Committee in Biomedical Research of the Dos de Mayo National Hospital and reviewed by the Ethics Committee of the Universidad Peruana Cayetano Heredia in December 2015.

3. Results

332 patients were evaluated, in four study groups 1 (HIV+ TB+) (59), 2 (HIV+ TB−) (91), 3 (HIV− TB+) (92) and 4 (HIV− TB−) (90). There was a predominance of males (89.83%, 82.61%, 63.04%, 44.44%), the median age and interquartile range of age was similar, being in the range of 25 - 35 years (Table 1).

The ESAT-6 protein in urine was present in all study groups, with TB patients having a higher concentration. Group 1 presented a median and interquartile range of 38.51 ± 46.98 ng/ml; group 3, 28.33 ± 25.56 ng/ml. The TB -control patients, group 2 14.06 ± 31.17 ng/ml and finally group 4 9.16 ± 27.98 ng/ml.

Regarding TB patients, within groups 1 and 3, there was a higher proportion of patients with pulmonary TB, 42 (71.19%) and 86 (93.48%), respectively. Of these, 20 (33.90%) and 57 (61.96%) showed positivity in smear microscopy, 26 (45.61) and 76 (83.52) in Ogawa culture and 15 (41.67) and 68 (74.73) in MODS culture. Sensitivity to drugs was only determined in 23 patients (38.98%) in the HIV+ participants' and in 78 participants (84.78%) in the HIV-group.

The median ESAT-6 concentration in urine has a statistically significant association with TB status (p = 0.00). The median concentration of ESAT-6 in urine did not show statistical association with the level of HIV viral load copies/ml (p = 0.45) and CD4 cells/ml (p = 0.26) in the HIV population (Table 2).

Table 1. Sociodemographic data.

| Grupo | VIH+ TB+ (59) | VIH+ TB− (91) | VIH− TB+ (92) | VIH− TB− (90) |
|-------|--------------|--------------|--------------|--------------|
| Sex   |
| Male  | 53 (89.83)   | 76 (82.61)   | 58 (63.04)   | 40 (44.44)   |
| Age*  | 33 ± 15      | 35 ± 15      | 29.5 ± 22.5  | 25 ± 15      |
| Ocupation |
| Student | 4 (6.77)     | 7 (7.69)     | 15 (16.67)   | 46 (51.68)   |
| Health Personnel | 1 (1.69) | 3 (3.30) | 0 (0.00) | 19 (21.35) |
| Employed | 46 (76.24) | 68 (74.73) | 65 (72.21) | 20 (20.22) |
| Unemployed | 9 (15.25) | 13 (14.29) | 10 (11.11) | 4 (4.49) |
| History of TB |
| Yes | 19 (32.76) | 22 (24.18) | 12 (13.33) | 4 (4.60) |
| ESAT-6 (ng/ml)* | 38.51 ± 46.98 | 14.06 ± 31.17 | 28.33 ± 25.56 | 9.16 ± 27.98 |

*Median and interquartile range.
### Table 2. HIV and TB status.

|                  | VIH+ TB+ (59)* | VIH+ TB− (91)* | p** |
|------------------|----------------|----------------|-----|
| CD4/mm³ cell level | 105 ± 214       | 311 ± 321       | 0.00 |
| HIV viral load copies/mL | 112,250 ± 396,800 | 403 ± 128,500 | 0.00 |
| ESAT-6 ng/ml     | 38.51 ± 46.98   | 14.06 ± 31.17   | 0.00 |

*Median and interquartile range, **Mann Whitney U test.

### Table 3. Sensitivity and specificity of the ESAT detection test using NIPAm according to subgroups.

|                                    | ROC   | Obs | IC          | Cutpoint | Sensitivity | Specificity |
|------------------------------------|-------|-----|-------------|----------|-------------|-------------|
| **TB all locations**               |       |     |             |          |             |             |
| Cutpoint ≥24.06                     | 71.72%| 147 | 0.63 - 0.80 | 71.92%   | 68.89%      |             |
| Cutpoint ≥37.17                     |       |     |             |          |             |             |
| Patients with HIV infection <200 CD4 cells - TB all locations |       |     |             |          |             |             |
| ROC                               | 73.51%| 76  | 0.61 - 0.85 | 79.55%   | 68.75%      |             |
| Cutpoint ≥21.78                    |       |     |             |          |             |             |
| Cutpoint ≥26.2                     |       |     |             |          |             |             |
| Cutpoint ≥29.50                    |       |     |             |          |             |             |
| Patients with HIV infection >200 CD4 cells - TB all locations |       |     |             |          |             |             |
| ROC                               | 70.28%| 83  | 0.58 - 0.83 | 72.00%   | 68.97%      |             |
| Cutpoint ≥24.6                     |       |     |             |          |             |             |
| Pulmonary TB                       |       |     |             |          |             |             |
| ROC                               | 75.15%| 128 | 0.66 - 0.84 | 78.95%   | 65.56%      |             |
| Cutpoint ≥21.78                    |       |     |             |          |             |             |
| Cutpoint ≥24.06                    |       |     |             |          |             |             |

A sensitivity and specificity analysis of the test was performed according to study subgroups. Thus, for the diagnosis of TB all locations in patients with HIV infection, an AUC of 71.72, sensitivity of 71.92%, specificity of 68.89% was determined, considering as a cut-off point ≥24.6 ng ESAT-6/ml of urine (Table 3).

When the population was divided into patients with and without AIDS phase (<200 CD4 cells/ml). In patients with a CD4 cell level <200 cells/ml, an AUC 73.51, sensitivity 70.45%, specificity 71.88% were determined considering a cut-off point ≥26.20 ng ESAT-6/ml of urine, considering 76 samples for analysis. In the group with CD4 cells > 200 cells/ml, AUC 70.28, sensitivity 72.00%, specificity 68.97% considering a cut-off point ≥24.60 ng ESAT-6/ml of urine, with 83 samples for analysis.

For the diagnosis of pulmonary TB, independent of CD4 cells, an AUC 75.15, sensitivity 78.95% and specificity of 68.89% were determined, with a cut-off point ≥24.60 ng ESAT-6/ml of urine, considering 128 samples to be analyzed.

### 4. Conclusions

For the study groups, AUC value was greater >70, sensitivity >60% and specificity >60% were found. The group with the best rates was for the diagnosis of pulmonary tuberculosis in patients with HIV infection.

Urine samples are easy and quicker to obtain, is only necessary refrigeration
as soon as possible. Is plausible method, with lower risk of handling potentially infectious samples. Likewise, by detection of a TB marker, decreases high exposure to health personnel by obtaining and managing samples like bronchial aspirates, biopsies, or induced in HIV patients.

In the study, two preanalytical procedures were analyzed for treated and untreated urine both calibration curves were validated. However, there was no significant difference between the calibration curves of both methods. The untreated urine protocol requires fewer preanalytical conditions and is more feasible for field application. However, in recent years, health centers have more equipment, making it possible to use a thermoblock and a centrifuge, where samples can be processed into pellets for transport in the laboratory. Although the nanoparticles are stable at room temperature and could be used in the field, the protocol for treated urine and its use in the laboratory allows us to include the initial solution (urine) and the supernatant in the immunoassay, which serves as an additional internal control of the test.

In the study, the development of the immunoassay was carried out in two equipment, the method was validated in both FluorChem R FR0001 and C Digit Blot Scanner equipment, however, a difference between the thresholds was detected. For the application of the test, the method must be validated using the available chemiluminescence reader.

The immunoassay procedure takes up to 12 hours, despite the duration of the procedure, it is considerably less than an incubation time of a culture and at a lower cost of a molecular test. More experiments are necessary to decrease the time in the procedures in order for the method to be more adaptable to the clinical field.

The sensitivity and specificity values of the NIPA ESAT-6 urine test are greater than 70%, more sensitive, less specific than smear microscopy (sensitivity 28%, specificity 92%) and less sensitive or specific than a culture (sensitivity 80% - 90%, specificity 98%) in the HIV population, all with an AUC value >73%, classifying it as a good test.

Several studies have tested the detection of new biomarkers in both sputum and urine, thus, in 2017, a study testing a cocktail immunoassay of antigens such as ESAT-6, CFP10 and MPT64 in urine, the sensitivities were low 68%, 2%, 22% and 31.6%, respectively [26].

In Indonesia, they carried out a qualitative detection study of antigens by immunochromatography such as ESAT-6, CFP-10 and MPT64, a 78% specificity and 68.8% sensitivity were determined, determining that the markers should more study [2].

In Geneva, performed a new electrochemiluminescence method for the detection of ESAT-6 in urine and serum, the method presented a minimum detection threshold as pg/ml. In urine, the sensitivity of LAM and ESAT-6 were 93% and 65%, respectively. For ESAT-6, 55% and 46% were presented, respectively [27].

Likewise, there is the Silva LAMP, for the detection of LAM for the diagnosis of extrapulmonary tuberculosis, which determined a sensitivity of 60% for Pul-
Pulmonary TB and 67% for Extrapulmonary TB. Although this test has presented good results, its validation is still under study. Various studies have studied the ability to detect ESAT-6 in urine and blood by different immunoassay methods, however, the sensitivity remains low. There is the possibility that the low levels of sensitivity are due to the low concentration in urine or the enzymatic degradation [28] [29] [30].

Due to this, in 2013, an immunoassay with gold nanoparticles, an immunoassay (ELISA) was carried out for the detection of ESAT-6 where promising results were presented, this type of nanoparticles presented a concentration factor of 7.5×. However, the hydrogel nanoparticles showed greater stability and a concentration factor of up to 50× [21].

The study method determined to be applicable for the diagnosis of TB in all locations, however, better rates were determined for the diagnosis of pulmonary TB, this possibly due to misclassification bias in patients with extrapulmonary TB due to the low sensitivity of the tests for the TB diagnosis and clinical assessment for initiation of treatment. To determine the sensitivity and specificity of the extrapulmonary TB test, confirmatory studies by molecular or microbiological biology with more sensitive methods are necessary, as well as a larger sample size. However, the results of the study allow us to say that this test is possibly even more sensitive than current conventional tests. There are molecular tests that have determined a sensitivity level greater than 40% in the non-HIV population. However, in a population with coinfection, it may have a lower level. As well as the difficulty of applying mPCR tests [25].

The present study of nanoparticles shows promising results of sensitivity, the test can be used as a TB screening that must be interpreted together with a clinical and epidemiological evaluation, thus reducing the delay in the start of treatment. However, larger studies of latent TB should be performed in the same population to eliminate possible classification bias.

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**Conflicts of Interest**

The authors declare no conflicts of interest.

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