Research Article

# Urine-Based Nested PCR for the Diagnosis of *Mycobacterium tuberculosis*: A Comparative Study Between HIV-Positive and HIV-Negative Patients

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## Abstract

**Background:** While tuberculosis (TB) can be diagnosed by microscopy and culture, the sensitivity of Ziehl-Neelsen staining is variable and culture results require 4 - 8 weeks to be determined. Polymerase chain reaction (PCR) and its modifications, including nested PCR, might be promising methods for the rapid diagnosis of TB.
**Objectives:** This study aimed to evaluate the performance of nested PCR on urine samples of human immunodeficiency virus (HIV)-positive and -negative patients with different manifestations of clinical TB.
**Methods:** In a prospective study, three early-morning urine samples from 100 patients with pulmonary TB (PTB) or extrapulmonary TB (EPTB) were evaluated using a molecular target with insertion element IS6110, specific to the *Mycobacterium tuberculosis* genome, and nested PCR was performed. The results were analyzed with SPSS version 22.
**Results:** A total of 100 patients, including 74 (74%) with PTB and 26 (26%) with EPTB, were enrolled. Positive smears were seen in 38 patients (38%). Lymph nodes were the most commonly involved organ in 14 of the 26 (53.8%) EPTB patients (13.5%). Seven (23.1%) of the EPTB patients were HIV-positive. Urine PCR was positive in only 28 patients (28%). Seven HIV-positive patients with PTB showed positive urine PCR results. Moreover, PCR results were positive in only one of the seven HIV-positive subjects with EPTB. Positive PCR results were found in 20 of the 73 HIV-negative patients (27.4%) and in 8 of the 27 HIV-positive patients (29.6%). Therefore, there was no significant difference between the HIV-negative and HIV-positive patients for urine PCR (sensitivity 29.6%, specificity 72.6%; positive and negative predictive values 28% and 72%, respectively; P = 0.138).
**Conclusions:** Nested PCR showed the same sensitivity in HIV-positive and HIV-negative patients. It can be applied as a rapid technique for the diagnosis of TB.

*Keywords:* Polymerase Chain Reaction, Urine Specimen Collection, HIV Seropositivity, *Mycobacterium tuberculosis*

## 1. Background

Tuberculosis (TB) is an infectious disease that affected approximately nine million individuals in 2013, killing an estimated 1.5 million. Roughly 13% of patients with TB are also infected with human immunodeficiency virus (HIV), and approximately one fourth of deaths from TB (360,000 cases) occur among HIV-positive patients. HIV infection is believed to increase the risk of TB by 26 - 31 times. In fact, TB is the most prevalent disease in patients with HIV and a major cause of their deaths, regardless of the administration of antiretroviral therapy. Fortunately, however, advances in the diagnosis and treatment of TB have resulted in a downward trend in its prevalence, preventing the deaths of over 37 million individuals during 2000 - 2013. Although TB has been reported in almost all parts of the world (202 countries and territories), roughly 56% of TB cases in 2013 were seen in Southeast Asia and the Western pacific region (1, 2). According to the national survey of Iran, an estimated 10,987 individuals developed TB in 2013, 3.8% of whom were HIV-positive. The incidence rate in 2011 was also calculated as 21 TB patients per 100,000 people (2).

Sputum smear microscopy has long been regarded as an inexpensive and popular method for the diagnosis of TB and for evaluation of the response to treatment. However, this method lacks adequate sensitivity, especially in HIV-positive individuals and in children, and fails to differ-

entiate the *Mycobacterium tuberculosis* complex from non-tuberculosis mycobacterium (NTM). Therefore, culturing is considered the standard method not only for differentiation between these two groups of mycobacteria, but also for the confirmation of drug-resistant TB. Despite its benefits, culturing is time-consuming and cannot be performed in the absence of highly trained personnel, a well-designed transport system, and an equipped laboratory (3, 4). On the other hand, the absence of clinical symptoms or abnormal findings on chest X-ray, along with negative acid-fast bacilli (AFB) smear results in HIV-positive patients with pulmonary TB (PTB) (5, 6), delays the diagnosis and treatment of active disease, leading to a poor prognosis in this group of patients. Furthermore, cytological and histological tests are essential for the diagnosis of extrapulmonary TB (EPTB). Simple non-invasive molecular methods can also be adopted to confirm the presence of *M. tuberculosis* complex and to facilitate the diagnosis of EPTB.

Over the past decade, extensive research has focused on the development of faster and more sensitive diagnostic methods to be used as either alternatives or supplements to conventional diagnostic tests for TB (e.g. culturing). Studies have confirmed the potential of molecular tests based on amplification of fragments of genomic sequences of *M. tuberculosis* to detect the bacillus in biological samples. Genomic detection by polymerase chain reaction (PCR) was also introduced as a rapid, simple, and very sensitive tool capable of detecting low concentrations (< 10 bacilli/mL) of mycobacterial species in clinical samples (7-10). Gholoobi et al. (11) compared the results of PCR and cultures in 30 different clinical samples from TB patients, and reported the sensitivity, specificity, and positive and negative predictive values of insertion sequence 6110 (IS6110)/buffer PCR to be 58.33%, 77.78%, 100%, and 78.26%, respectively. Several other studies have also evaluated the sensitivity and specificity of PCR using different samples (12-14). Although conventional PCR is very useful for the detection of *M. tuberculosis*, the use of two sets of primers, as in nested PCR, would definitely enhance both the sensitivity and the specificity of the method (15). While limited research has examined the accuracy of urine testing for the detection of *M. tuberculosis*, IS6110/buffer single-tube nested PCR has been recommended as a method to detect the presence of this mycobacterial species in urine samples (16, 17).

## 2. Objectives

The present study sought to compare the sensitivity of PCR with that of smears and cultures for detecting *M. tuberculosis* in urine samples of HIV-positive and -negative patients with active TB in different organs.

## 3. Methods

This study involving 100 patients with a diagnosis of *M. tuberculosis* PTB or EPTB was carried out during September 2013 - 2014 at the TB control center of Bandar Abbas health center, Iran. Clinical signs, along with microbiological (AFB smear-positive) and pathological findings, were used to classify microbiological positivity or negativity of PTB and EPTB patients. First, three morning urine specimens were pooled and centrifuged at 3,000 g and 4°C for 20 minutes. The method described by Torrea et al. (18) was adopted for mycobacterial DNA extraction. At the final stage of the process, the DNA obtained from ethanol precipitation was resuspended in 40 $\mu$L of distilled water. Afterward, nested PCR, based on the amplification of a region of the 16srRNA gene that is conserved among mycobacterial species, was performed using two sets of primers (Fermentas, Germany). The outer primers, which yielded a 439-bp product, were KY75: 5-GCCCGTATCGCCCGCACGCTCACA-3 and KY18: 5-CACATGCAAGTCGAACGGAAAGG-3. The inner primer pairs, which yielded a 123-bp product, were T4: 5-CCTGCGAGCGTAGGCGTCGG-3 and T5: 5-CTCGTCCAGCGCCGCTTCGG-3. The thermal cycling conditions consisted of 40 cycles at 94°C for one minute followed by 60 cycles at 72°C, each for one minute. A pre-denaturation step was also performed at 95°C for five minutes.

For the second reaction, 2 $\mu$L of the first PCR products were mixed with 48 $\mu$l of the PCR mixture. The thermal cycle conditions were similar to those of the first-round PCR. However, the number of amplification cycles was increased to 45. Ultimately, agarose gel electrophoresis (using 2% gel in 1 $\times$ Tris-acetate-ethylenediaminetetraacetic acid [TAE] buffer) and ethidium bromide staining were applied to analyze the PCR products. Positive control DNA for *M. tuberculosis* was extracted from PTB cultures. Sensitivity, specificity, and positive and negative predictive values were calculated in SPSS 22.0 (SPSS Inc., Chicago, IL, USA) with the use of McNemar's and Pearson's chi-square statistical tests.

## 4. Results

A total of 100 patients, including 68 men and 32 women, were enrolled in the present study. The mean age of the patients was 43.1 $\pm$ 17.7 years. PTB and EPTB were present in 74 (74%) and 26 (26%) of the subjects, respectively. AFB smear-negative 36 (36%) and smear-positive 38 (38%) PTB subjects constituted 14% and 37% of the participants, respectively (Table 1). Thirteen smear-positive

and 6 smear-negative PTB patients had urine PCR positivity (sensitivity 34.2%, specificity 83.3%; P = 0.001); there was significant difference between smear-positive and smear-negative urine PCR results. The rest of the patients had abnormal chest X-rays or were culture-positive. Lymph nodes were the most-involved organ in the subjects with EPTB (14%). Of the 27 HIV-positive patients, eight (29.6%) also had positive PCR results. Urine PCR was positive in only 28 patients (28%). Moreover, among the 38 smear-positive patients, positive and negative urine PCR results were seen in 13 (34.2%) and 25 (65.8) of the patients, respectively (Table 2).

Urine PCR had no significant correlations with type of TB. Since only 28 fresh cases of TB exhibited positive urine PCR, this method cannot be helpful in determining fresh cases of the disease (P > 0.05). Positive urine PCR was found in seven HIV-positive patients with PTB and one HIV-positive patient with EPTB (sensitivity 16.7%, specificity 66.7%; P = 0.93). Therefore, the urine PCR value in the diagnosis of PTB or EPTB in HIV-positive patients is the same. Meanwhile, 20 of 73 (27.4%) HIV-negative patients had positive PCR results and eight (29.6%) of 27 HIV-positive patients had PCR positivity (sensitivity 29.6%, specificity 72.6%, positive and negative predictive values of nested PCR 28% and 72%, respectively; P = 0.138). Therefore, urine PCR does not have a higher sensitivity in the diagnosis of TB in HIV-positive compared to HIV-negative patients.

## 5. Discussion

The rapid initial diagnosis of an *M. tuberculosis* infection is problematic if the techniques of direct visualization are negative. The definitive diagnosis depends on culture of the mycobacterium, a technique that is time-consuming and not always sensitive. PCR is a rapid, yet costly, alternative method for the diagnosis of *M. tuberculosis*. It not only accelerates the diagnosis of *M. tuberculosis*, but may also enhance the detection of mycobacteria in smear-negative patients. Studies on PCR have reported different sensitivity and specificity values. The present study compared the sensitivity of PCR with that of smear and culture in the diagnosis of *M. tuberculosis* in HIV-positive and -negative patients. da Silva et al. (19) reported the sensitivity, specificity, accuracy, and positive and negative predictive values of PCR in HIV-negative patients with PTB as 64%, 74%, 68%, 75%, and 63%, respectively.

The corresponding values in HIV-positive patients were 59%, 33%, 56%, 87%, and 10%. In contrast to our findings, da Silva et al. (19) found PCR to have a better performance in HIV-negative patients than in HIV-positive individuals, but in our study there was no difference between HIV-positive

and HIV-negative patients with PTB. The sensitivity, specificity, and positive and negative predictive values of PCR in HIV-positive and -negative patients with PTB were 35%, 76.5%, 26.8%, and 73.2%, respectively. In a study on pleural effusion, Montenegro et al. (20) observed sensitivities of 84.2%, 72.2%, and 33.3%, respectively, when nested PCR was applied to (i) pleural fluid, blood, and/or urine samples; (ii) blood and/or urine samples without pleural fluid samples; and (iii) pleural fluid samples alone. They concluded that the sensitivity of the test could be improved by the use of different samples.

Considering the simplicity of urine sample collection, the present study used urine PCR analysis to test 100 patients with PTB and EPTB. The results indicated the applicability of PCR for the rapid detection of *M. tuberculosis* in urine from patients with or without HIV infection. In fact, by centrifugation of urine samples and DNA amplification, different concentrations of mycobacteria could be detected in individuals with PTB and EPTB. Nevertheless, this technique achieved the same performance in HIV-positive and -negative subjects. Due to its high sensitivity, PCR analysis of urine may be able to detect mycobacterial infections even before the clinical manifestation of the disease.

Studies on the use of urine samples for TB diagnoses among HIV-positive and -negative individuals are scarce. Although Torrea et al. (18) attempted to examine the performance of nested PCR in the analysis of urine from individuals with suspected TB, they did not use any bacteriological measures to confirm the PCR results and determine the positive predictive value of urine samples compared to other types of specimens. According to available literature, the application of conventional microbiological tests on urine will not be beneficial for the detection of *M. tuberculosis*, except in patients with genitourinary disease (21, 22). In a previous study, DNA amplification suggested the presence of *M. tuberculosis* in blood from 39 out of 41 patients with confirmed PTB. Therefore, since mycobacteria can enter the circulation even before the clinical manifestation of the infection, they may be excreted through the urine (23).

Aceti et al. (17) examined 13 HIV-positive patients with confirmed active PTB. While all subjects tested positive for *M. tuberculosis* using urine-based nested PCR, cultures detected only two cases, and acid-fast staining yielded negative results for all samples. Hence, although *M. tuberculosis* might exist in the urine of all TB patients, or at least those who are HIV-positive, its low concentration in urine may prevent conventional methods for its accurate detection. Torrea et al. (18) calculated the overall sensitivity of PCR in HIV-positive and -negative patients with microbiologically-positive PTB, microbiologically-negative PTB, and EPTB as 40.5%, 66.7%, and 57.1%, respectively. They also reported a

**Table 1.** Distribution of Participants Based on Smear, PCR, HIV, and Type of TB

| Value | No. (%) |
|---|---|
| **Male** | 68 (68) |
| **Female** | 32 (32) |
| **HIV positive** | 27 (27) |
| **PCR-positive** | 28 (28) |
| **PTB** | 74 (74) |
| **Smear-positive** | 38 (38) |
| **Smear-negative** | 36 (36) |
| **HIV-negative** | 73 (73) |
| **EPTB** | 26 (26) |
| **Lymph nodes** | 14 (14) |

Abbreviations: HIV, human immunodeficiency virus; PCR, polymerase chain reaction; TB, tuberculosis; PTB, pulmonary TB; EPTB, extrapulmonary TB.

**Table 2.** Distribution of Participants According to Smear, PCR, and HIV Seropositivity

| Value | PCR (No.) | Positivity (%) |
|---|---|---|
| **AFB smear-positive/HIV-positive** | 3 | 50 |
| **AFB smear-positive/HIV-negative** | 10 | 31.3 |
| **AFB smear-negative/HIV-positive** | 4 | 26.7 |
| **AFB smear-negative/HIV-negative** | 2 | 9.5 |
| **PTB** | | |
|     HIV-positive | 7 | 33.3 |
|     HIV-negative | 14 | 66.7 |
|     Total | 21 | 100 |
| **EPTB** | | |
|     HIV-positive | 1 | 16.7 |
|     HIV-negative | 6 | 83.3 |
|     Total | 7 | 100 |
| HIV-positive (n = 27) | 8 | 29.6 |
| HIV-negative (n = 73) | 20 | 27.4 |

Abbreviations: AFB, acid-fast bacilli; HIV, human immunodeficiency virus; TB, tuberculosis; PTB, pulmonary TB; EPTB, extrapulmonary TB.

specificity of 98.2%. Our findings indicated lower sensitivity in patients with PTB (25.6%) and EPTB (42.3%). Consequently, while PCR may not be a favorable method for the routine detection of *M. tuberculosis* in patients with new TB, it can be beneficial for confirming the presence of *M. tuberculosis* in suspected cases of PTB or EPTB with negative microbiological test results and inconclusive clinical and bacteriological diagnoses.

Irrespective of the type of biological sample, nested PCR had the same sensitivity in HIV-positive and -negative individuals. Therefore, despite its fairly undesirable sensitivity in some cases, this rapid technique can serve as a beneficial tool, particularly in HIV-positive patients and those for whom the conventional diagnostic methods fail to yield a confirmatory diagnosis. Future studies are recommended to examine the performance of PCR-based techniques by using other types of biological specimens, such as blood and urine. The incorporation of nested PCR in the diagnostic approach to detecting *M. tuberculosis* will facilitate the early diagnosis and timely management of this bacterial infection.

## Acknowledgments

## Footnotes

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