

Full Length Research Paper

Comparison of Nested-PCR technique and culture method in detection of *Mycobacterium tuberculosis* from patients suspected to genitourinary tuberculosis

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Accepted 26 February, 2010

The aim of the present study was to evaluate the diagnostic value of nested PCR in genitourinary tuberculosis (GUTB) compared with acid fast staining and culture method. In total 200 urine samples from suspected cases of GUTB were collected during the period of study. Urine pellets were used for smear preparation, culture and DNA extraction by ether-chloroform method. Nested PCR was performed according to standard protocol using primers based on IS6110 gene fragment. The results obtained by PCR were compared with those obtained by standard acid-fast bacilli stain and culture method. Based on obtained results, the positivity rate of urine samples in this study was 5.0% by using culture and PCR methods and 2.5% for acid fast staining. Four out of total samples showed positive results in all three methods (2%). The sensitivity of PCR in this study was estimated as high as culture equal to 100%, while the sensitivity for direct smear staining was 41.6%. In conclusion, the obtained rate of GUTB in our study was 5.0%. Since the detection rate of culture and nested PCR was identical, we could suggest PCR as a rapid alternative to culture especially for confirmed cases of GUTB.

Key words: Genitourinary, nested PCR, culture, *Mycobacterium tuberculosis*.

INTRODUCTION

Mycobacterium tuberculosis (MTB), the etiologic agent of tuberculosis (TB), is estimated to have infected one-third of the world's population and annually causes ~8 million new TB cases and >2 million deaths (Corbett et al., 2003). Tuberculosis is still a major health hazard in both developed and developing countries (Kafwabulula et al., 2002). Although MTB is mainly affecting the lungs, however, kidneys are the second target organ for the

bacterium (Lenk et al., 2001). According to a recent report, EPTB constitutes up to 20% of the total cases of the disease, and with the involving rate of 14%, the urogenital system is one of the most common affected sites (Yazdani et al., 2008). According to Warren et al. (2002) statement, "While renal TB is uncommon in developed countries, as many as 15 to 20% of TB patients in developing countries are found with *M. tuberculosis* in the urine". The infection almost always affects the kidneys during the primary exposure to infection but does not present clinically. The course of renal tuberculosis may be indolent, with the appearance of few, if any, symptoms. Presentation is usually late and symptoms usually occur as a result of nonspecific urinary tract infection (Nawaz and Chandramohan, 2004). Genitourinary TB (GUTB) is usually caused by metastatic spread of organisms through the blood stream during the initial infection. Active disease results from the reactivation of the initial infection (Cek et al., 2005; Warren et al., 2002).

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Abbreviations: TB, Tuberculosis; GUTB, genitourinary tuberculosis; PCR, polymerase chain reaction; MTB, *Mycobacterium tuberculosis*; EPTB, extrapulmonary tuberculosis; AFB, acid-fast bacilli; SDS, sodium dodecyl sulfate; CTAB, cetyltrimethyl ammonium bromide; EDTA, ethylene diamine tetraacetic acid; RNase, ribonuclease.

Table 1. *M. tuberculosis* specific primers used in present study (Githui, 1999).

Primer	Sequence
Outer primers	
Tb294	5'-GGACAACGCCGAATTGCGAAGGGC-3'
Tb850	5'-TAGGCGTCGGTGACAAAAGGCCACG-3'
Inner primers	
Tb505	5'-ACGACCACATCAACC-3'
Tb670	5'-AGTTTGGTCATCAGCC-3'

The spread to the kidneys usually is hematogenous from the lungs, bone, or a gastro-intestinal tract focus. The true incidence of renal tuberculosis may be underestimated since radiological findings may be absent and diagnosis is made by urine culture. Genital tuberculosis is usually secondary to renal tuberculous infection (Nawaz and Chandramohan, 2004).

Despite the global importance of tuberculosis, the diagnosis of EPTB in its different clinical presentations remains a true challenge. The disease's underestimation by clinicians and the use of insensitive conventional analytical methods have contributed to the difficulties in managing patients with EPTB (Gomes et al., 2000).

Among the EPTBs, the diagnostic criterion for GUTB is the isolation of MTB from urine. This is not easy to achieve, as the discharge of organisms into the urine is sporadic and, more importantly, involves few organism (Hemal et al., 2000). The conventional method for diagnosing TB using clinical samples by the acid-fast bacilli (AFB) smear has low sensitivity and specificity and culture for MTB is time consuming (Lima et al., 2008).

Due to the difficulties associated with diagnosing GUTB, there has been considerable interest in applying PCR methods for the detection of these diseases. Polymerase chain reaction (PCR) is a technique used to amplify extremely small amounts of a specific genomic sequence rapidly. The presence of an extremely small number of bacteria can thus be detected within 24 to 48 h (Hemal et al., 2000).

This study was designed to evaluate the sensitivity and specificity of the nested-PCR method for the diagnosis of GUTB in patients showing suspicious clinical findings suggesting GUTB and comparing the results with conventional methods of acid fast microscopy and culture.

MATERIALS AND METHODS

In total, 200 urine samples from suspected cases of GUTB were collected from January to October 2007. For each patient, three urine samples collected on three consecutive days as early morning urine. The specimens were pooled and centrifuged at 3000 g for 20 min. Supernatant was removed and the pellet was divided into two part. One part was used for acid fast staining by the Ziehl-Neelsen method and culture on Lowenstein-Jensen medium and for the grown bacteria conventional identification procedure was used

(Forbes et al., 2007). Thirty milliliters PBS was added to the second part of the urine pellets and were centrifuged at 3000 g for 20 min. The pellets were re-suspended in 1 ml PBS, and aliquots of 500 μ l were stored at -20°C until analysis. DNA extraction was performed by the method of phenol chloroform as described previously (Kafwabulula et al., 2002). In brief, the urine pellet suspended in 1 \times TE buffer (10 mM Tris-HCL plus 1mM EDTA, pH 8.0). Then 20% sodium dodecyl sulfate (SDS) and 20 mg/ml proteinase K (Cinnagen co., Tehran, Iran) were added to the pellet, mixed well and incubated at 37°C for one hour. The extract was RNase treated at a concentration of 10 mg/ml RNase, 5 M NaCl, CTAB/NaCl. DNA was purified with phenol-chloroform and precipitated with 100% isopropanol. The pellet was washed with 70% ethanol, dried and re-suspended in 30 μ l of 1 \times TE buffer, and stored at -20°C until use. The study was approved by the Ethical Committee of the AJUMS. Standard precautions were taken to prevent cross contamination of DNA.

The urine samples were subjected to nested PCR method described previously by Githui et al. (1999) using MTB specific primers as shown in Table 1.

The composition of PCR mixture was 50mM KCl, 10 mM Tris-HCl (pH, 8.3), 1.5 mM MgCl₂, 200 mM dNTPs, 0.5 μ M of each primer, 2 U of *Taq* polymerase and 5 μ l of DNA template in a final volume of 50 μ l. All the reagents were purchased from Cinnagen Company, Tehran, Iran. The standard *M. tuberculosis* H37Rv (Institute Pasteur, Iran), was used as positive and a few non-mycobacterial strains were used as negative controls.

Using a thermocycler (Techgene, UK), conditions for first round PCR (regular) were as 94°C for 3 min as initial denaturation, followed by 30 cycles of 94°C for 45 s for denaturation, 62°C for 30 s for annealing, 72°C for 45 s for extension, and a final extension of 72°C for 5 min. Second round PCR (nested) was performed with the same cycling program as regular PCR, except for extension time which reduced to 30 s. The amplified products were analyzed by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. Product sizes were identified using a 100 bp DNA ladder (MBI Fermentas, Germany) as a reference standard. Results were recorded using the gel documentation system (UVP Systems, UK).

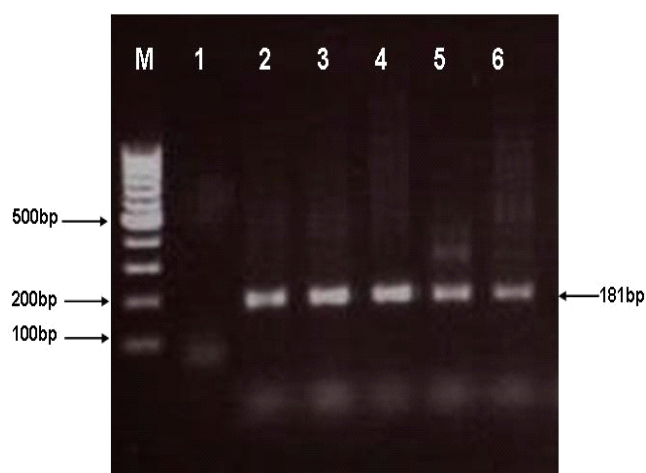
RESULTS

Of the 200 patients, 70 were male and 130 were female with mean age of 37.8. Out of these, 11 patients showed positive results with methods used. Among patients with positive culture results, four were male and seven were females.

The nested PCR, culture, and smear examination results obtained with the urine samples are shown in Table 2. As shown, in one patient with positive culture, PCR

Table 2. Results obtained with acid fast staining, culture and nested PCR in present study.

Patient	Smear	Culture	PCR	Isolated organism
1	+	+	+	MTB*
2	+	+	+	MTB*
3	+	+	+	MTB*
4	+	+	+	MTB*
5	-	+	+	MTB*
6	-	+	+	MTB*
7	-	+	+	MTB*
8	-	+	+	MTB*
9	-	+	+	MTB*
10	-	+	+	MTB*
11	-	+	-	NTM**

**Figure 1.** Gel electrophoresis of the Nested-PCR amplification using *M. tuberculosis* specific primers. **Lanes:** **M**, molecular size marker; **1**, negative control; **2**, positive control; **3-6**, positive samples.

could not detect *M. tuberculosis* and this was later identified as a case of nontuberculous mycobacteria.

Among the patients five cases had a history of anti-TB therapy, which with the exception of one case with a weak positive smear, others showed negative results in all three methods used. Besides, three patients had a history of previous GUTB but none of their samples were positive by the methods used. The positivity rate of urine samples in this study was 5.0% by using culture and PCR methods. Four out of total samples showed positive results in all three methods (2%). Positive results were obtained for PCR in all six cases with positive culture. We ignored one of the positive cultures which later showed the identification criteria of nontuberculous mycobacteria, so the sensitivity of PCR in this study was estimated as high as culture equal to 100%. Figure 1 shows an example of the nested PCR amplification. The sensitivity for direct

smear staining was 41.6% in this study. All the patients with positive specimens were 41 years old and above.

DISCUSSION

Although GUTB is an uncommon form of TB, however it is considered as a severe form of EPTB (World Health Organization, 2003). The diagnostic criterion for GUTB is the isolation of MTB from urine. This is not easy to achieve, as the discharge of organisms into the urine is sporadic and is limited to a few organisms (Moussa et al., 2000).

Acid fast-staining has low sensitivity for detecting GUTB; direct smears are often negative and do not differentiate tuberculous from nontuberculous mycobacteria. Mycobacterial culture method which is more sensitive, takes 6 to 8 weeks because of the slow growth rate of mycobacteria (Gomes et al., 2000; Torrea et al., 2005).

In present study, we investigated urine samples of the patients with GUTB to evaluate the diagnostic potential of nested PCR in rapid diagnosis of GUTB. The obtained prevalence of GUTB in our study was 5.0% based on the results from culture and PCR both. This was lower compared to that reported by Indian Council of Medical Research on 2240 patients suspected of having urinary tract tuberculosis, which the incidence of a positive urine culture for MTB was 10.7% (Colabawalla, 1990).

Moussa et al. (2000), in a study on 1000 urine specimens, reported a sensitivity of 95.59 and 52.07% for PCR and AFB staining, respectively, compared with culture. In our work, the sensitivity of culture and PCR was identical and was slightly higher, however, the sensitivity of our acid fast staining was lower than those.

Aslan et al. (2007), examined urine samples collected from 437 patients. The detection rate of MTB in their study was 3.4% for acid fast staining and 3.8% for culture which our findings were close to their work. This was

lower in our survey for acid fast staining as 2.5%, however in culture, our rate was higher. In a study on confirmed cases of GUTB, the MTB detection rate was reported as 80.95% for PCR, 37.14% for culture and 30.95% for acid fast staining (Hemal et al., 2000). More recently, Dochviri et al. (2005) used PCR for detection of MTB in 32 patients with confirmed GUTB. MTB in urine has been detected in 26 (81.25%) patients under investigation.

In a recent study from Iran, using 33 patients with confirmed diagnosis of urinary tuberculosis, a detection rate of 48.5% for PCR, 57.6% for culture and 18.2% for acid fast staining were reported (Yazdani et al., 2008).

In such studies with higher rates of MTB detection, the patients were confirmed GUTB cases, while in our investigation, the studied patients were suspected and unconfirmed cases. We believe that we are still weak in screening of cases with GUTB in our clinical sectors, and many patients are referred to TB reference laboratory without real suspicious to GUTB. This may well explain the lower detection rate of MTB in present study. Besides, a few patients were under confirmed anti-TB therapy prior to laboratory attendance and for some others there was no access to a history of therapy. Moreover, the mycobacterial diseases including GUTB are normally more common in individuals with immunosuppression. There are reports of higher incidence rates of GUTB in AIDS (Torrea et al., 2005; Figueiredo et al., 2009) and hemodialysis patients (Chuang et al., 2003; Queipo et al., 2003). According to the information on patients in present study, none of them belonged to these two groups.

All the patients with positive culture and PCR were 40 years old and above. Based on the other statements, there is often a latent period of 20 years or more between infection with the tubercle bacillus and the expression of GUTB (Cek et al. 2005). Due to this, GUTB is rare among people under the age of twenty five, and these patients are more likely to have a family history positive for TB (Ferrie et al., 1985). This well explains the prevalence of GUTB in older age patients in present study.

The sensitivity of culture and nested PCR was identical in our study as 100%, ignoring one positive culture which the organism was identified as NTM by further tests. This specimen showed negative PCR result, since we have used the specific primers for MTB and obviously; NTM was not detected by such primers. Besides, using strains of nontuberculous mycobacteria as negative controls guaranteed the specificity of our nested PCR. We also had five patients under anti-TB therapy that none of their specimens showed positive results except for one positive case by acid fast staining. Obviously at least for the latter patient, we could have a positive result by culture and PCR in the absence of therapy and thus the sensitivity of our methods would be increased.

In conclusion, the detection rate of GUTB was low in our study. We believe that the PCR method is more reliable when it is used for confirmed cases of GUTB by

culture or for patients with strong clinical suspicion of GUTB. However, based on our results we could suggest PCR analysis of urine samples in condition of well optimization, as a valid alternative for fast and sensitive detection of *M. tuberculosis* DNA as other investigators have been shown (Kafwabulula et al., 2002; Lima et al., 2008).

ACKNOWLEDGEMENTS

This study was financially supported by a grant from the research affairs, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (approved no. U-87011). We gratefully thank to Khuzestan TB Reference Laboratory staff, PHLS, Ahvaz, Iran, for their help in providing the samples and preliminary works.

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