Comparison of culture and microscopic methods by PCR for detection of *Mycobacterium tuberculosis* in sputum

Ahmad Khorshidi, Mahdi Rohani[*], Rezvan Moniri, Mahnaz Torfeh

Department of Microbiology, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran

ABSTRACT

Background: It is difficult to diagnose *Mycobacterium tuberculosis* infection due to a lack of rapid, sensitive, and specific tests. Newer methods, which are easy and reliable, are required to diagnose TB at an early stage. Our aim is to evaluate the polymerase chain reaction (PCR) technique, using primers directed against the IS6110 gene, for the detection of *M. tuberculosis* in the sputum samples, and calculate the sensitivity and specificity of PCR.

Patients and methods: A total of 248 sputum samples from patients suspected of mycobacterial diseases were studied. DNA was extracted by boiling method. IS6110 PCR method by a specific pair of primers designed to amplify 123bp and 245bp sequences of the insertion sequence, 6110, in the *M. tuberculosis* genome was used to analyze sputum samples.

Results: Totally, 32 (12.9%) samples had positive culture. PCR yielded a sensitivity of 93.8**%** and specificity of 99.1% for the diagnosis of TB, when diagnosis was confirmed by culture. There were 2 out of 32(6.3%) PCR-positive cases among the patients with non-TB disease.

Conclusion: We concluded that the performance of an IS6110 PCR assay is valuable in the rapid diagnosis of tuberculosis.

Keywords: *Mycobacterium tuberculosis, Diagnosis, Polymerase chain reaction*.
(Iranian Journal of Clinical Infectious Diseases 2009;4(4):228-232).

INTRODUCTION

Tuberculosis is one of the most hideous infectious diseases which was declining for some decades, but is now on the rise alongside the appearance of AIDS. The World Health Organization estimates that if stronger measures are not taken up to control the prevalence of this disease, from 2000 to 2020 a billion people will be infected by the bacterium, 200 million of which will develop the disease and 35 million people will expire as a result. Currently, there are approximately 8 million new infections and 3 million deaths attributed to *M. tuberculosis* each year (1,2). Therefore, the WHO has declared tuberculosis a universal emergency. At the present time, the diagnosis of tuberculosis relies on Ziehl-Nielson staining and bacterial culture on Lowenstein-Johnson medium, taking 6-8 weeks. Using various techniques of PCR and multiplex PCR for detection of bacteria in clinical samples has aided the researchers and clinicians greatly (3). The gold standard in detecting *M.tuberculosis* is culture, considered the standard method by all references.

Several rapid diagnostic modalities for the identification of mycobacteria have been

developed. The major advance was the detection of *M. tuberculosis* by nucleic acid amplification tests (NAT). These technologies are attractive because of the possibility of directly detecting *M.tuberculosis* in clinical respiratory specimens. PCR-based assays for the detection of *M. tuberculosis* approach the sensitivity and specificity of conventional culture but have the added advantage of being rapid (4). Unfortunately, because of the possibility of false-negative reactions among other reasons, the use of NAT for *M. tuberculosis* has come under scrutiny and their usefulness has been questioned (5). In addition, the Centers for Disease Control and Prevention (CDC) has suggested an algorithm for the use of these tests that includes repeat testing, which is costly and unattractive to the laboratory (5).

Various loci have been utilized to diagnose *M. tuberculosis*, such as insertion sequence 6110, antigens 65KDa (Gro EL), 38 KDa (PhoS) and 23 KDa (MPB64) (6). Among these, PCR of the IS6110 sequence showed the highest sensitivity compared to PCR of other gene loci (7). The repetition of this sequence increases the sensitivity of PCR which involves the amplification of a single DNA sequence. In other words, the single DNA has multiple segments to be proliferated, and thus the chance of primer binding increases even with small amounts of DNA. Furthermore, this segment occurs only in *M. tuberculosis* complex, and this is another advantage for using it in detecting *M. tuberculosis* (8,9). In some regions like India, strains lacking this segment have been reported, however many studies have never reported the lack of this segment in regions like Brazil (10,11).

Considering the importance of rapidly diagnosing tuberculosis and the controversy concerning the sensitivity and specificity of PCR, we conducted this study to evaluate the sensitivity, specificity, positive predictive value, and negative predicative value of PCR in patients suspected of tuberculosis referring to Moslem Ben Aqil center, Kashan ,Iran, from May 2007 to August 2007.

PATIENTS and METHODS

This predictive value study was conducted on 248 sputum samples from patients suspected of tuberculosis referring to Moslem Ben Aqil center, Kashan, Iran, from May 2007 to August 2007.The sputum samples were digested and disinfected using the standard procedure (12). Subsequently the samples were used for Ziehl-Nielson staining, culture and PCR. All the samples were cultured on Lowenstein Johnson medium and kept for eight weeks in 37°C, and the positive cultures were biochemically identified. The DNA in sputum samples was extracted through boiling. In this procedure, each sample was centrifuged twice at $10000g$ and the precipitate was dissolved in 1mL sterile distilled water. Then, the solution was heated for 20 minutes at 85°C to simultaneously kill bacteria and extract DNA. In this study, one sample of *M. tuberculosis* (clinically isolated strain) was used as positive control.

The PCR reaction was optimized by varying outer primer annealing temperature and primer concentration. The PCR was performed in 50μl of reaction mixture containing 10mM Tris-HCl (pH 8.4), 50mM KCl, 2.5mM $MgCl_2$, 15pmol each of primer and optimal concentration of dNTPs (SinaGENE, Tehran, Iran) and 1μl of 50 unit Taq DNA polymerase (SinaGENE, Tehran, Iran) and 5μl of DNA extracted from sputum samples.

The reaction mixture was initially denatured at 94°C for 5 minutes, and subjected to 35 cycles of PCR comprising denaturation at 94ºC for 45 seconds, annealing at 68ºC for 45 seconds and extension at 72ºC for 45 seconds. Finally, the PCR products were detected by electrophoresis of 5μl reaction mixture in 1.2% agarose gel and examination of the ethidium bromide-stained gel under a UV transilluminator (INGENIUS, SYNGENE).

The primers for IS61610 included M1: 5´CCTGCGAGCGTAGGCGTCGG3´ and M2: 5´CTCGTCCAGCGCCGCTTCGG3´ for amplifying the 123 base pair segment, and C1:5´GCGTAGGCGTCGGTGACAAA3´and C2: 5´CGTGAGGGCATCGAGGTGGC3´ for amplifying the 245 base pair segment (figure 1).
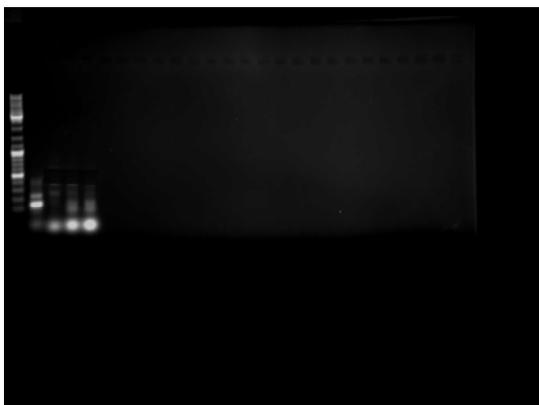
Figure 1. Amplification of DNA template isolated from sputum specimens using multiplex PCR. PCR was performed with isolated DNA of sputum specimens (lanes 2). The amplified product is shown as band of 123 bp and a band of 245 bp. Lane 1 is 100 bp DNA marker.

RESULTS

Of 248 sputum samples, 40 samples (16.1%) showed acid fast bacteria after Ziehl Nielson staining (positive smear) and 32 samples (12.9%) were culture positive on Lowenstein Johnson medium.

Table 1. Detecting *Mycobacterium tuberculosis* in sputum samples using multiplex polymerase chain reaction (PCR)

In this study, the occurrence of each band was considered positive PCR. Totally, 32 samples (12.9%) had positive PCR. Table 1 compares the culture, smear and PCR results. Table 2 presents the sensitivity, specificity, and negative and positive predictive values of PCR in comparison with culture.

Table 2. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of polymerase chain reaction (PCR) and smear compared to culture

	Sensitivity	Specificity	PPV	NPV
PCR	93.8	99.1	93.8	99.1
Smear	100	96.2	80.0	100

DISCUSSION

Other studies from different regions of the world report 80-90% sensitivity for PCR, considering various DNA extraction methods (7). In our study all samples with positive smear and culture (except 6) had positive PCR result. From 32 cases with positive PCR, 30 cases (93.75%) were corroborated by culture, and 22 cases (64.45%) were corroborated by both smear and culture. The sensitivity of the *M. tuberculosis* complex PCR ranged from 40.6% (95%CI:34.0–47.4) in microbiological positive pulmonary TB (PTB) patients to 66.7% (95%CI:47.1–82.1) in microbiological-negative PTB patients. The specificity of the test was 98.2% (95%CI:89.0–99.9) in all categories. The PPV of the test was well above 95% in all categories. The NPV was much lower with values ranging from 29.5% (95%CI:23.1–36.8) in microbiological-positive PTB patients to 84.4% (95%CI:72.7–91.9) in microbiological-negative PTB patients (13). Querol et al (14) tested 314 respiratory samples using IS6110 primer and reported a sensitivity of 97% compared to culture. Thoe et al (15) in Singapore used IS6110 PCR and Amplicor to report sensitivity and specificity of 86.5% and 83.6%, respectively. In this study, the small amount of samples, using unequal samples, and lack of IS6110 segment are reasons that prevent the detection of *M. tuberculosis*. Christiane Schewe et al (16) showed that the highest sensitivity was achieved for the amplification of the IS6110 gene followed by the 16S rDNA of *M. tuberculosis*

complex, whereas regarding specificity, the latter was superior to the IS6110 detection.

In our study, we evaluated the performance of a test based on PCR amplification in sputum using the IS6110 sequence of *M. tuberculosis* complex as a target to be amplified. While evaluating the reproducibility of our *M. tuberculosis* complex PCR technique, we found that some samples had negative PCR results despite of positive culture (tow samples), this suggests that *M. tuberculosis* complex DNA is represented in low copy numbers and/or is unevenly distributed in sputum samples, as already suggested by others (17,18).

In the light of these results, *M. tuberculosis* complex PCR should be recommended for routine detection of new TB patients in Kashan. The technique may, however, be useful for confirming *M. tuberculosis* in patients suspected to be microbiological-negative PTB when clinical and bacteriological diagnoses are not conclusive.

Rapid diagnosis of tuberculosis may have profound effects in patients' care. These effects may be considered from different aspects. In smear-positive patients with positive PCR, the etiology of the disease would be tuberculosis bacillus, and the patient must be cared for until the smear turns negative; but a negative PCR may indicate non tuberculosis mycobacterial infection; thus the patient must no longer undergo *M. tuberculosis* isolation procedures, and proper therapy should be initiated. The potential effects of this method in reducing the therapeutic costs are evident.

It may be concluded that nucleic acid amplification methods corroborate positive smears, and in patients with clinically suspicious symptoms but negative smears, may account for the presence of *M. tuberculosis*. The recent guidelines of CDC verify this conclusion (19). In cases of negative smear, it is recommended to withhold initiation of therapy until the culture results are ready, because this condition bears a low risk of disease transfer. PCR, in diagnosis of tuberculosis, provide rapidity

in gain of results but sensitivity and specificity of this method has not shown difference from smear and culture. In addition to the rapidity of molecular methods, such methods may be used directly in clinical and veterinary laboratories to enhance public health. Moreover, they may be helpful in diagnosing extra pulmonary infections of tuberculosis, particularly tuberculotic meningitis (20).

The addition of specific primers for different species of mycobacterium, especially *M. bovis*, multiplex PCR may be used to distinguish various species of mycobacterium which cause diseases in man or animals.

ACKNOWLEDGEMENT

REFERENCES

1. Sohn KY, Shrestha S, Khagi A, Malla SS, Pokharel BM, Khanal MP, et al. Polymerase chain reaction detection of Mycobacterium tuberculosis from sputum. Journal of the Nepal Medical Association 2003;42:65-70

2. Maher D. The internationally recommended tuberculosis control strategy. World Health Organization. Trop Doct. 1999;29(3):185-6.

3. Mahaisavariya P, Chaiprasert A, Manonukul J, Khemngern S, Tingtoy N. Detection and identification of mycobacterium species by polymerase chain reaction (PCR) from paraffin-embedded tissue compare to AFB staining in pathological sections. J Med Assoc Thai. 2005;88(1):108-13.

4. D'Amato RF, Wallman AA, Hochstein LH, Colaninno PM, Scardamaglia M, Ardila E, et al. Rapid diagnosis of pulmonary tuberculosis by using Roche AMPLICOR *Mycobacterium tuberculosis* PCR test. J Clin Microbiol 1995;33:1832–34.

5. Centers for Disease Control and Prevention. Update: nucleic acid amplification tests for tuberculosis. MMWR Morb Mortal Wkly Rep 2000;49:593–94.

6. Gengvinij N, Pattanakitsakul S, Chierakul N, Chaiprasert A. Detection of *Mycobacterium tuberculosis* from sputum specimens using one-tube nested PCR. Southeast Asian J Trop Med Public Health 2001; 32(1):114-25.

7. Ogusku MM, Salem JI. Analysis of different primers used in the PCR method: diagnosis of tuberculosis in the state of Amazonas, Brazil. Journal Brasileiro de Pneumologia. 2004;30(4): 433-39.

8. Kaul KL. Molecular detection of *Mycobacterium tuberculosis*: Impact on patient care. Clin Chem. 2001;47:8:1553–58.

9. Ghazvini K, Sani AT, Rastegari S, Ghanaat J, Behdani M. Detection of rifampin resistant tuberculosis meningitis by polymerase chain reaction linked single strand conformation polymorphism analysis of cerebrospinal fluid. Neurosciences. 2007;12(2):105-8.

10. Salem JI, Maroja MF, Carvalho FF, Lima MO, Feuillet A. Mycobacterium other than tubercule bacilli in sputum specimens from patient in Manaus Amazonas, Brazil. Acta Amazonica. 1989;19:349-54.

11. Salem JI, Gentijo Filho PP, Frebault VL, David HL. Isolation and characterization of mycobacteria colonizing the healthy skin. Acta Leprologia. 1989; 7:18-20.

12. Kent P, Kubica G. Public health mycobacteriology: A guide for the level III laboratory. Atlanta, Ga: US Department of Health and Human Services, Centres for Disease Control, 1985.

13. Torrea G, Van de Perre P, Ouedraogo M, Zougba A, Sawadogo A, Dingtoumda B,et al. PCR-based detection of the Mycobacterium tuberculosis complex in urine of HIV-infected and uninfected pulmonary and extrapulmonary tuberculosis patients in Burkina Faso. J Med Microbiol. 2005;54:39–44.

14. Querol JM, Farga MA, Granda D, Gimeno C, Garcia-de-Lomas J. The utility of polymerase chain reaction in the diagnosis of pulmonary tuberculosis. Chest. 1995;4:1631-35:

15. Thoe SY, Tay L, Sng EH. Evaluation of amplicor and IS6110 PCR for direct detection of *Mycobacterium tuberculosis* complex in Singapore. Trop Med Int Health. 1997;2(11):1095-101.

16. Schewe C, Goldmann T, Grosser M, Zink A, Schlüns K, Pahl S, et al. Inter-laboratory validation of PCR-based detection of *Mycobacterium tuberculosis* in formalin-fixed, paraffin-embedded tissues. Virchow's Arch. 2005;447:573–85.

17. Kafwabulula M, Ahmed K, Nagatake T, Gotoh J, Mitarai S, Oizumi K, et al. Evaluation of PCR-based methods for the diagnosis of tuberculosis by identification of mycobacterial DNA in urine samples. Int J Tuber Lung Dis. 2002;6:732–37.

18. Jouveshomme S, Cambau E, Trystram D, Szpytma M, Sougakoff W, Derenne JP, et al. Clinical utility of an amplification test based on ligase chain reaction in pulmonary tuberculosis. Am J Respir Crit Care Med. 1998;158:1096–101.

19. Centers for Disease Control and Prevention. Update: nucleic acid amplification tests for tuberculosis. MMWR Morbidity Mortality Weakly Report. 2000;49:593-4.

20. Kaul KL. Molecular detection of *Mycobacterium tuberculosis*: Impact on patient care. Clin Chem. 2001;47(8):1553-58.