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Research Article

THE PREVALENCE OF *MYCOBACTERIUM TUBERCULOSIS* (TB) IN RESPIRATORY AFFECTED AFGHANI HABITANTS DETECTED BY POLYMERASE CHAIN REACTION (PCR) TECHNIQUE, ISFAHAN, IRAN

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ABSTRACT
The prevalence of *Mycobacterium tuberculosis* was assessed via efficient polymerase chain reaction (PCR), using the insertion sequence IS6110 as target for DNA in respiratory affected Afghani habitants hospitalized patients in Shahid Sadughi Hospital, Isfahan, Iran. A total of 40 patients were evaluated. Cases were defined by specific clinical criteria. PCR was done on the sputum and compared with clinical findings, radiological features and Acid Fast Bacteria (AFB) staining. The sensitivity of PCR in pleural fluid samples was 100% but the sensitivity of AFB staining was found to be 20%. PCR technique is a valuable diagnostic tool for the diagnosis of tuberculosis (TB) and the prevalence of affected Afghani was found to be 75%.

***Key Words*:** *Mycobacterium Tuberculosis, Polymerase Chain Reaction (PCR), Afghani Patients, Acid Fast Bacteria (AFB) Staining, Iran*

INTRODUCTION
Tuberculosis (TB) has been remained a major cause of morbidity and mortality throughout the world in both industrialized and developing countries in spite of availability of antitubercular therapy (ATT) for many years. In 1990, majority of TB occurred in Asia and Africa with an increasing number among HIV infected individuals (Sudre, Tendam & Kochi, 1992). Diagnosing this disease in affected patients is required a sensitive, specific and rapid method of diagnosis because clinical symptoms are not apparent and more than 50% cases show no symptoms at all at the time of presentation (Snider et al., 1998).
Conventional diagnostic tests, including acid-fast bacteria (AFB) staining and mycobacterial culture, are frequently negative in patients with TB disease because of lower M. tuberculosis counts. Also, *M. tuberculosis* culture takes a longer time, which limits its usefulness in the initial diagnosis (Smith et al., 1996).
Detection by DNA amplification through polymerase chain reaction (PCR) has developed a promise to identification of pathogens, which grow with difficulty or take a long time to grow. This has enabled early detection of mycobacterium DNA directly from clinical specimens (Mullis and Faloona, 1987). PCR using insertion sequence IS6110 as the target, has the potential to go beyond the limitations of conventional procedures and be established as a rapid, sensitive and specific technique for detecting DNA of *M. Tuberculosis* in various clinical specimens (Patel et al., 1990; Shankar et al., 1990). IS6110 has been said to be specific to *M. tuberculosis* as most strains of *M. tuberculosis* have between 8 and 15 copies (Hermans et al., 1990a; McAdam et al., 1990; Noordhoek, van Embden and Kolk, 1996). Therefore we evaluated the prevalence of TB by PCR as a tool to diagnosis TB at Afghani patients in Isfahan, Iran.

MATERIALS AND METHODS
From March 2003 to May 2004, patients aged 10-60 years old admitted in Shahid Sadughi Hospital, located in Isfahan, Iran were selected for the study. The assays were performed in the Pathology Laboratory, where a PCR laboratory had been set up for diagnosing cases of *M. tuberculosis*. Forty

Afghani patients suspected to be suffering from TB, but radiological features did not show anything and 8 patients were detected as positive AFB staining. Their symptoms were low grade fever, loss of appetite, failure to gain weight or loss of weight, and two-week cough.

Extraction of DNA (Buch, O'Hara and Summersgill, 1992)

Boiling the sputum and 200 ml of sample was mixed with 200 ml of lysis buffer (5.3 M Guanidine isothionate, 10 mM Diethyribiol, 1% Tween 20, 0.3 M Sodium acetate, 50 mM Sodium citrate) and 50 ml proteinas K the mixture was incubated at 65° C for 10 minutes. Thereafter, it was centrifuged at 1200 g for 1 minute. Supernatant was discarded and matrix re-suspended in wash buffer (50% ethanol, 10 mM Tris HCl, 100 mM NaCl). Bound DNA was eluted by periodic mixing in 100ml of 10 mM Tris HCl.

Primers

The insertion sequence IS6110 was the target for the PCR. Primers T4, T5 were selected for amplification of a 123 base pair nucleotide sequence in IS6110. The sequence was as follows:

Primer 1: T4-5' CCT GCG AGC GTA GGC GTC GG 35'

Primer 2: T5'-55' CTC GTC CAG CGC CGC TTC GG 35'

The buffers, Taq polymerase enzymes and custom synthesized primers were all obtained from Roche Co. and used for PCR.

Amplification of DNA

PCR reaction was done in 25 ml vol. Reaction master contained 10X reaction buffer (2.5 ml/sample), dNTPs (200 ml/sample), forward and reverse primers (1 ml/sample) and Taq polymerase enzyme (1 unit/sample). Amplification was carried out on a Cambridge Techne thermo-cycler with a heated lid. The hot start method was employed at 94° C for 2 minutes initially. Thereafter, amplification was carried out for 35 cycles at -90° C for 1 minute (denaturation), 60° C for 1 minute (annealing) and 72° C for 10 minutes. The amplification products were analyzed on 2% agarose gel. The bands were visualized by staining with ethidium bormide under ultraviolet light. Positive and negative controls were run with each batch or sample analyzed.

The following precautions were taken to avoid false negative cases in the study (13): (a) Use of guanidine isothiocyanate in the lysis buffer, during pre-treatment of samples for DNA extraction; (b) Testing of several specimens; and (c) All specimens were concentrated by centrifugation before proceeding for analysis.

RESULTS

A total of 40 Afghani patients were evaluated, 8 cases were positive with AFB staining. Thirty cases, of probable TB were positive for PCR and test repeated after 10 days did not show any change. All 30 cases of pleural effusion were positive with PCR but *M. tuberculosis* was grown on culture from only 9 specimens while in the other specimens there was no yield on culture. Eight of the samples showed AFB on smear. Ten cases of probable pulmonary TB showed pulmonary infiltrates on radiological examination. Out of the two positive cases, one had cavitations in radiological examination of chest. All cases were given ATT and improved.

DISCUSSION

Tuberculosis even today, remains a major health problem especially in poor countries. The problem is further complicated by the fact that the conventional epidemiological criteria used for assessment (prevalence and incidence of infection, prevalence of suspected and drug resistant cases and mortality) are difficult to apply in this category because of the absence of an objective method or a "gold standard" for diagnosis of tuberculosis (Seth, 1991). Positive smears are rarely obtained but amplification of *M. tuberculosis* specific DNA sequences in clinical samples is the most sensitive and rapid method of detection available (Delacourt et al., 1995; Hermans et al., 1990b). PCR has a sensitivity as 88%-100% (Tan et al., 1997; Schluge et al., 1994) and specificity as more than 90% (Tan et al., 1997; Eisenach et al., 1991).

We detected mycobacterial DNA after amplification by PCR in clinical samples from 30 Afghani patients with respiratory affected diagnosis based on clinical, investigative parameters and response to treatment although none of the samples yielded *M. tuberculosis* on culture. PCR could obtain an exact high prevalence for TB among Afghani patients. Various authors have shown a sensitivity ranging from 40% to 100%. Monno et al. (1996) showed 100% sensitivity with PCR. Culture results form sputum samples in these studies were very poor. Our results were similar, indicating that PCR is extremely detecting *M. tuberculosis* in sputum from patients with TB (Shankar et al., 1991; Monno et al., 1996; Kaneko et al., 1990).

In our study, 2 false positive results were obtained from control samples. False positive results are known to be a problem with PCR technique, both, in research and hospital laboratories (Seth, 1991; Noordhoek et al., 1994). There are two recognized explanations for false positive PCR results. The most common is the carryover of amplicons from previous reactions (even when the laboratory activities are well separated from each other). Another source is cross contamination with *Mycobacterium tuberculosis* DNA isolated from positive clinical samples during the processing procedure. The frequency with which these problems occur is difficult to document (Smith et al., 1996). Also in our study, considering all the samples evaluated, sensitivity was 100% pleural fluid specimens. Specificity of PCR in our study was 94% (2 false positive out of 39) but owing to small number of control samples this figure may be misleading. However, Delacourt et al (1995) have shown a specificity of 100% , while Smith et al (1996) have shown 80% specificity in their studies.

The result of various studies shows that PCR can detect very low levels of AFB in the clinical specimens with specificity ranges from 80-100% (Smith et al., 1996; Delacourt et al., 1995; Singh 1991). PCR results can be revealed within 3 days as compared to 3 weeks with culture but with its greatest weakness as even the smallest amount of contaminating DNA can be amplified resulting in misleading results. Therefore, through some precautions 100% true specificity with PCR is possible via collecting samples in two volumes, one of which is separately stored for later use if necessary and processing a small number of samples at one time (Shankar et al., 1991).

The limitations of our study were as follows: (1) the specimens were small in number, hence the specificity and prevalence results can be misleading; (2) more number of patients of pulmonary and extrapulmonary TB need to be evaluated before usefulness of this technique can be conclusively proved. Through concluding our results, it is elucidated that prevalence of TB is high among immigrant Afghani habitants in Isfahan, Iran and many of them may be transmitter for TB; therefore, a health policy should be developed for prevention, diagnosis and treatment TB among these people. Also, the utility of PCR for diagnosing TB in Iranian conditions is promise. However, an integrative procedure including PCR, epidemiological, clinical, radiological, tuberculin testing, and response to treatment should be planned as the criteria of diagnosis of TB.

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