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REVALIDATION OF SMEAR NEGATIVE TUBERCULAR INFECTION BY CONVENTIONAL AND PCR APPLICATIONS

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Abstract: *IS6110* sequence based Polymerase Chain reaction (PCR) was compared with conventional bacteriological techniques in the laboratory diagnosis of *Mycobacterium tuberculosis* (MTB). A retrospective study involving one hundred and twenty six, non-repeated clinical isolates patients being investigated for tuberculosis. The samples were also processed for Ziehl-Neelsen (ZN) staining for acid fast bacilli (AFB) and culture for MTB. All the samples were processed for PCR amplification with primer targeting 123 bp fragments of insertion sequences *IS6110* of *M. tuberculosis* complex (MTC) and the sensitivity of PCR was analyzed. Of the 126 patients, 100% and 97.3% were smears and culture positive for MTB respectively. Using culture as the gold standard, the overall sensitivity of PCR was 97.62%, and for either positive or either negative clinical isolates it was 97.06%% and 92.31%, respectively. The current study evaluated a PCR assay for the detection of MTC strains by targeting the IS*6110* insertion element. This PCR has emerged as a rapid, reliable and a potent tool in establishing the diagnosis of tuberculosis with higher sensitivity than ZN microscopy and greater alacrity than culture.

Key Words: *IS6110*, *M. tuberculosis,* smear positive, culture positive, polymerase chain reaction

INTRODUCTION

Tuberculosis (TB), one of the oldest and evergreen human infections, is still one of the biggest killers among the infectious disease and disaster for medical science. The emergence of Drug resistance (DR) has been found in all affected countries and in new areas [1]. An extremely worrisome aspect of MTB is the recent rise in multi-drug resistant (MDR) MTB cases in several countries [2,3]. Directly-observed treatment short course (DOTS), the strategy endorsed by the WHO, is effective in preventing the emergence of DR; however, in practice, only 27% of TB patients actually receive DOTS [4].

With the global rise of human Immunodeficiency virus infection (HIV), MTB accounts for more than 50% cases of TB among HIV positive patients [5]. The prevalence of MDR also underscores the immediate need for novel strategies to combat the pathogen. Currently, the routine diagnosis of TB is based on positive smear for acid fast bacilli (AFB) and culture of MTB. Direct smears lack sensitivity and cultures take at least two to six weeks with additional weeks for diagnosis of drug resistance [6]. Such delayed diagnosis facilitates further transmission of MTB. A quick and reliable diagnosis can permit early chemotherapeutic intervention and hence interrupt transmission. This could improve the prognosis, quicker diagnosis and appropriate therapies in controlling this growing epidemic [7, 8, 9, 10].

In developing countries, the diagnosis of MTB with conventional diagnostic methods is a greater challenge. Smear for AFB is reported to be positive in less than 53 to 87% of patients and *Mycobacterium* culture is reported positive in variable proportions [6, 10, 11,12]. In clinical practice, anti-tuberculosis treatment is often started purely on the basis of compatible clinical symptoms and signs, suggestive radiological changes, and a rapid microbiological test result, either a positive smear for AFB, and/or positive PCR for TB [13, 14]. Confirmation of TB by positive culture often takes three to six weeks. Even before the availability of culture results, patients may have clinical deterioration, which could be related to an alternative non-tuberculosis infection, side effects of anti-tuberculosis treatment and by anti-tuberculosis drug resistance.

Recent advances in the field of molecular biology and progress in the understanding of the molecular basis of DR in MTB have provided new tools for its rapid diagnosis by molecular methods [9, 10, 15].

Molecular diagnostic methods such as conventional PCR, which are faster than culture diagnosis, have variable success in the diagnosis of tuberculosis [16, 17, 18]. Some reports have evaluated the role of PCR is the diagnosis of MTB and in Extra-Pulmonary Tuberculosis (EPTB) with high sensitivity and specificity using various primers to amplify targets like *IS6110*, 65Kda, TRC4 dev R etc. [11, 14, 19-23]. The results of PCR testing can help to speed up the decision making process involved in the diagnosis of TB, so that early anti-tuberculosis treatment can be initiated. The laboratory performance of both commercial and in house TB PCR in the diagnosis of TB has been extensively evaluated [9, 10, 24-28].

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Pulmonary TB (PTB), the most important type of TB from the public health point of view, can be diagnosed by its symptoms, chest radiography, sputum smear microscopy, and by cultivation of MTB. A percentage of patients, however, are not confirmed bacteriologically and are only diagnosed on the basis of high clinical suspicion and response to anti-TB drugs [29]. In some cases, the diagnosis of TB becomes even more problematic due to several factors associated with immune-suppression in patients as it occurs in HIV infected persons or in the case of latent infection or EPTB. Due to its nonspecific clinical presentation, diagnosis of TB is also problematic in children [30].

The present study of 126 patients was undertaken to evaluate the, clinical significance of *IS6110* gene based PCR assay in clinically suspected and smear negative cases to diagnose MTB cases; its comparison with conventional bacteriological techniques and in the initiation of anti-tuberculosis treatment.

MATERIALS AND METHODS

Patients

Our study was performed at King George's Medical University (KGMU), UP, Lucknow from 1st September 2012 to 30 August 2014. During the study period, all patients referred to infectious disease consultation with clinical features suggestive of TB were recruited into our study. The cases of both sexes, age varied from 18 to 62 years, were residents from the peripheral region of Uttar Pradesh attending OPD of KGMU, UP.

Microbiological investigation for MTB

We performed investigations for MTB, including AFB smear examination and culture, on relevant clinical specimens of all patients with suspected TB. PCR was performed on PTB in the following clinical settings: (1) patients with typical radiological changes compatible with PTB; and (2) patients with radiological changes of old TB but presenting with new pulmonary infiltrates.

Microbiological processing of clinical Isolates

Three consecutive morning sputum samples from each patient were collected in properly labeled screw cap disposable plastic bottles after oral gurgling with normal water. Sputum samples were decontaminated and digested by treatment with an equal volume of sputolysin / sodium hydroxide (4%) for 30 minutes at room temperature. After neutralization with 20 ml of 0.067M sodium phosphate buffer (pH 5.3), the mixture was centrifuged and about 50µl of the sediment was inoculated into two tubes of Lowenstein-Jensen (LJ) medium and incubated at 37°C in automated culture system for up to six-eight weeks. Solid medium slants were considered positive when visible colonies grew. The colonies were further confirmed as mycobacteria by the Ziehl-Neelsen (ZN) stain [31, 32]. The rest of the sediment was transferred to an eppendorf tube and stored at –20°C till further use.

Extraction of DNA from clinical samples

The mycobacteria were cultured in LJ medium for 4-6 weeks. The cells were harvested, and chromosomal DNA was extracted by an enzymatic lysis method [33, 34]. The bacteria were pelleted by centrifugation and re-suspended in a 10mM Tris-HCl-1mM EDTA buffer (pH 8.0) [34]. Cell walls were digested with Lysozyme (10mg/ml), Proteinase K (10mg/ml), and 10% SDS. DNA was extracted using 0.3 M cetyltrimethylammonium bromide (CTAB) and 5M NaCl, purified by Phenol chloroform extraction. DNA was precipitated by adding 1 volume of isopropanol to the aqueous supernatant. After 30 min incubation at -20°C the mixture was centrifuged for 15 min at 10000 X g, the pellet was washed once with 70% ethanol, air-dried and finally suspended in Mili Q water [35].

PCR Amplification and documentation

Amplification of DNA was performed with primers IS-F5'CCTGCGAGCGTAGGCGTCGG3'and IS-R-3'CTCGTCCAGCGCCGCTTCGG5', to amplify 123 bp fragments of insertion element *IS6110* of MTB complex as reported earlier with some modifications [20, 36]. Briefly, PCR was performed using an automated gradient thermal cycler (Bio-Rad) and all reaction buffers contained 10 mM Tris/HC1 (pH 8.3), 50mM KC1, 1-5mM $MgCl_2$, 0.2 mM of each dNTP (Fermentas, U.S.A), 2-5 units Taq polymerase (Fermentas, USA), 1.0 µM of each primer, and 100ng template DNA in a final volume of 100µl. The amplification profile consisted of a denaturation step at 95°C for five minutes, followed by 30 cycles with denaturation at 95°C for one minute, primer annealing at 65°C, for one minute, and extension at 72°C for one minute. The PCR products were electrophoresed through 1.5% Agarose gels and stained with ethidium bromide. Visualization was done on a UV light illuminator (Chemidoc). The copy number of the amplified products was inferred from the difference between the molecular weights of the amplified products of the samples and those of the H37Rv strain. The length of the amplified products was used to compare with standard molecular weight markers (Fermentas, USA). The results were evaluated in the light of the performance appropriate positive control, to avoid false positive reactions. The sensitivity of the conventional tests and PCR Assay was calculated keeping each one of them as the gold standard against another.

RESULTS AND DISCUSSION

Patient's Diagnosis

Preliminary diagnosis of MTB was based on a positive AFB smear and their growth on LJ slants during the initial investigation. Specific identification and definitive diagnosis of TB is accomplished by the positive culture and PCR results.

One hundred and twenty six patients with negative smear and culture for MTB and clinical signs suggestive of TB were recruited into our study. Twenty nine were smear positive and 37 were culture positive for MTB in 126 patients. Both smear and culture for AFB was positive in 34. Twenty six out of 126 samples showed negative results by both Smear and culture. The diagnostic sensitivity of the AFB smear was 23% (29 of 126) and 29.37% (37 of 126) in culture confirmed and clinically diagnosed TB, respectively. Whereas, the diagnostic sensitivity of both the smear and culture positive was 26.98% (34 of 126), and smear and culture negative was 20.63% (26 of 126) (Table 1).

Table 1: Details of Pulmonary Clinical isolates investigated

No.	ZN Smear positive	AFB culture positive	Either positive	Either negative
1	29/126	37/126	34/126	26/126
2	23.02%	29.37%	26.98%	20.63%

Microbiological investigation for MTB

Table 2 shows the correlations between PCR and AFB smear and culture results. Twenty nine patients with a positive smear and 37 positive cultures for MTB had 100% and 100% PCR respectively. Another 26 patients had a negative smear and 24 positive PCR in the initial investigation, but only culture was subsequently positive in 37 patients. Those patients with a positive PCR but a negative culture had clinical and radiological results that were suggestive of active TB. PCR was positive in 37 of 126 patients who were culture positive for MTB. The diagnostic sensitivity of PCR was 100% (29 of 29), 100% (37 of 37), 97.06% (33 of 34) and 97.62% (24 of 26) in AFB smear positive, culture confirmed and clinically diagnosed TB, either positive or either negative respectively. The overall sensitivity of the PCR assay was 97.62% (Table 2). The contribution of PCR was observed high among samples that were negative by both the conventional bacteriological techniques- smear and culture for AFB (Table 3).

Table 3: Comparison of results of conventional bacteriological tests and PCR assay

S.No.	Conventional tests	No. of clinical isolates	PCR positive No. (%)
1	ZN smear positive	29	29 (100%)
2	Culture positive	37	37 (100%)
3	Either positive	34	33 (97.06%)
4	Both negative	26	24 (92.31%)

Table 2: The correlation between PCR and conventional bacteriological tests in clinical isolates.

No.	PCR Positive /ZN Smear positive cases	PCR positive /Culture positive cases	PCR positive /Either positive cases	PCR positive /Either Negative cases	Total PCR positive /Total samples
1	29/29	37/37	33/34	24/26*	123/126
2	100%	100%	97.06%	92.31%	97.62%

Analysis of PCR results

The correlation between PCR for MTB and conventional bacteriological test for MTB is 97.62% (123 of 126). Those patients with positive PCR but negative culture results had clinical and radiological results that were suggestive of active TB. 02 samples showed negative result in PCR. The remaining patients with negative results in both the AFB smear and PCR were investigated further and an alternative diagnosis was made (Fig.1).

The gold standard for TB diagnosis is the cultivation of MTB. It can be performed on a variety of specimens, such as sputum and bronchial washings, and also other non-pulmonary samples. It is much more sensitive than microscopy and it allows the recovery of the bacteria for other studies, such as drug susceptibility testing and genotyping. In some cases, the diagnosis of TB becomes even more problematic due to several factors associated with immune-suppression in patients as it occurs in HIV infected persons or in the case of latent infection or extra pulmonary tuberculosis (EPTB)[14, 27, 28].

This study suggests that PCR based identification is an attractive and rapid test for the diagnosis of clinically suspected /smear negative cases because *IS6110* sequence is present in most clinical isolates of MTB. PCR based amplification, using primer specific to *IS6110* was indicative of positive diagnosis of MTB of all the 126 cases studied (Fig. 1).

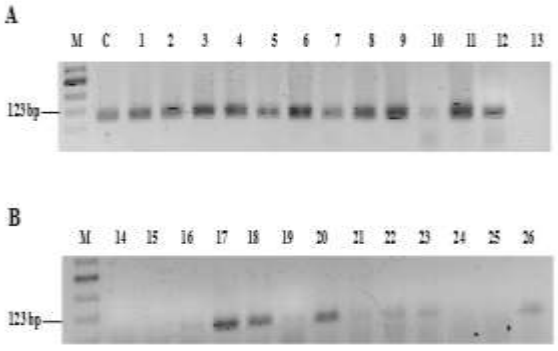

Figure 1. Primer IS*6110*, M-DNA Ladder, C-Control, Lane 1-26 Clinical isolates.

PCR based identification revealed that when conventional tests were taken as the gold standard; the sensitivity of the PCR assay was 97.62%, whereas when PCR assay was taken as gold standard, the sensitivity of conventional test was 23.02% and 29.37%. This suggests that among suspected cases, the PCR assay was more sensitive when compared to conventional tests (Fig. 1). It was more sensitive when compared to conventional tests in long treated cases where sputum productivity is less; PCR is being increasingly used in the diagnosis of TB. PCR either confirms or rejects the diagnosis when compared to conventional culture method, which takes about 4 to 8 weeks. It may be used to diagnose infection in adults and children with PTB who do not expectorate sputum [9, 14, 21, 25].

Among those samples found negative by conventional bacteriological techniques, the contribution of PCR was observed high (Table 2). The PCR assay was more sensitive when compared to conventional tests in long treated cases where sputum productivity is less and the methodology of PCR for *IS6110* has been widely carried out in the different technical set ups and has been proven to be simple and reproducible, compared to methodologies for PCR targeting other gene sequences [10, 12, 23].

Although the Food and Drug Administration of the USA recommended that PCR should only be performed for rapid diagnosis in respiratory specimens of either AFB smear positive or negative samples [37]. Nucleic acid amplification has been studied extensively in patients with EPTB using either in house or commercial kits [9,14]. The sensitivity ranged between 42% and 93% in culture positive specimens [9, 14, 37, 38].

MTB including MDR encounter many problems like pauci-bacillary nature of the samples, the inadequate sample amount or volume, processing of the samples for various diagnostic tests results in un-uniformed distribution of microorganisms etc. All these limitations reflect on the poor contribution of conventional bacteriological techniques in the establishment of a diagnosis of MTB. This has stimulated the application of PCR in the laboratory diagnosis of MTB [22, 39-43]. The decision to initiate anti-tuberculosis treatment based on the pretreatment nucleic acid amplification assay on PTB was studied. Without this rapid assay, delay in giving the appropriate treatment would probably have occurred

Our PCR assay was based on the amplification of a fragment of *IS6110*, Which is specifically for the MTB Complex [12, 20, 23]. The amplification of *IS6110* Insertion sequence, which belongs to IS3 family, is found in almost all members of MTB complex. Most strains of MTB carry 10-15 copies, which are present in a wide variety of chromosomal sites [44,45].

In our study, conventional bacteriological techniques were positive in 100 samples (AFB Positive, culture positive and either positive), whereas PCR shown 123 samples positive, out of total 126 samples processed (Table 2). Earlier studies also documented increased positivity by PCR targeting *IS6110* elements in clinical isolates of MTB [6, 10, 12, 26] and detection of MTB DNA in 57% of AFB negative MTB samples [7,14,22].

The overall sensitivity of the PCR assay was 97.23%, for pulmonary specimens, which is similar to that seen in another center using *IS6110* as a target site for laboratory diagnosis [23, 26].

DNA fingerprinting of MTB has been shown to be a powerful epidemiological tool because it exploits variability in both the no. and the genomic position of insertion sequences (IS) and tandem repeats (VNTRs) to generate strain specific patterns Although absence or the presence of fewer copies of the target sequence *IS6110*, in some strains of MTB has been reported [22, 23, 39, 42]. Some of the earlier studies also supported that PCR assays targeting *IS6110* sequence were more sensitive [39 - 41, 43]. Among samples found negative by conventional techniques, PCR targeting *IS6110* has shown higher positivity (26%) than PCR for other targets [9, 11, 12].

Although *IS6110* based PCR appears to be a rapid, sensitive, and specific diagnostic assay, the results should be interpreted with care in the clinical setting. In our study, 24 patients had a positive PCR but a negative culture subsequently (Table 2). In such cases, there is a clinical dilemma of whether antituberculosis treatment should be maintained or discontinued. As long as precautions are taken to avoid cross contamination, the positive PCR may indicate the presence of non-viable AFB, particularly in patients with a history of TB in the past. However, a negative AFB culture cannot preclude a clinical diagnosis of TB, particularly in patients with clinical and radiological features suggestive of active TB. There can be a variety of reasons for a positive PCR but negative AFB culture [46]. Therefore, PCR can be used to complement the culture in selected patients. Thus, it may be used to diagnose infection in adults and children with PTB who do not expectorate sputum and provide a tool to monitor the therapeutic efficacy.

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